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AN EVALUATION OF THE INTERTIDAL OLIGOCHAETE *PONTODRILUS BERMUDENSIS* BEDDARD AS DIETARY SUPPLEMENT FOR STIMULATION OF REPRODUCTION IN *PENAEUS SEMISULCATUS* DE HAAN

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OCTOBER 2001

DECLARATION

I here by declare that this Thesis entitled **"AN EVALUATION OF THE INTERTIDAL OLIGOCHAETE *PONTODRILUS BERMUDENSIS* BEDDARD AS A DIETARY SUPPLEMENT FOR STIMULATION OF REPRODUCTION IN *PENAEUS SEMISULCATUS* DE HAAN"** has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar titles.

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CERTIFICATE

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सारांश

प्रग्रहण स्थिति में पेनिआइड झींगों के पालन की मुख्य कठिनाई *अंडाशय* परिपक्वता तथा अंडजनन पर ठीक जानकारी प्राप्त करना है . वर्तमान अध्ययन में अपरक्षरण नहीं किए गए *पेनिअस सेमीसल्केट्स* (हरित पुलि झींगा) , जिसको अंतर तरंगीय ओलिगोकीटे *पोन्टोड्रिल्स बर्मुडेन्सिस* एक पूरक खाद्य के रूप में खिलाया गया है, में पुनरुत्पादकीय स्वभाव का मूल्यांकन किया जाता है . *पी. बर्मूडेन्सिस* से खिलाए गए *पी.सेमीसल्केट्स* के टेस्ट ग्रूप की तुलना सिर्फ स्क्विड तथा सीपी से खिलाए गए नियंत्रित ग्रुप से की गई . तब नियंत्रित ग्रुप की अपेक्षा टेस्ट ग्रुप के पुनरुत्पादन स्वभाव में उल्लेखनीय प्रगति देखी गई . कुल खाद्य का लगभग 9% कृमि का घटक होने पर अधिकतम प्रौढता देखी गई . *पी. बर्मुडेन्सिस* में होने वाले जैव रासायनिक मिश्रण एवं फैटी आसिड प्रोफाइल का विश्लेषण किया गया . *पी.बर्मुडेन्सिस* की बढती की तीनों अवस्थाओं में नोनक्लिटल्लेट्स को ब्रूड स्टॉक को खिलाने का अत्यंत अनुयोज्य जैव रासायनिक मिश्रण देखा गया . *पी.बर्मुडेन्सिस* में होनेवाले फैटी आसिड प्रोफाइल एन 6 फैटी आसिड विशेषत : सी 20:4 एन 6 की प्रचुरता व्यक्त हो गयी जो झींगा की प्रौढता और अंडजनन में सहायक प्रोस्टाग्रान्डिन्स के उत्पादन के लिए प्रमुख स्थान निभाता है . जल के अतिरिक्त भू स्थानों में ओलिगोकीटे का पालन विकसित करने के लिए दीर्घकालीन परीक्षण आयोजित किए गए . परीक्षण की गई तीन पालन व्यवस्थाओं में से यह व्यक्त हो गया कि लकडी की पेटियों में समुद्र तट की रेत और गोबर तथा समुद्री शैवाल का मिश्रण या गोबर और पत्ते कार्बनिक अमेन्डमेन्टों के रूप में दिए जाने पर *पी. बर्मुडेन्सिस* की अच्छी बढती होती है . झींगा परिपक्वन व्यवस्थाओं के एक भाग के रूप में कृमियों के वितरण के लिए कम लागत की सुविधा से एक बहु एकक की व्यवस्था जिससे क्रमिक संग्रहण और कार्बनिक वस्तुओं का वितरण किया जा सके, का प्रस्ताव है .

***पेनिअस सेमीसल्केट्स* डी हान का पुनरुत्पादन उत्तेजित करने के लिए अंतर तरंगीय ओलिगोकीटे *पोन्टोड्रिल्स बर्मुडेन्सिस* बेड्डार्ड पूरक खाद्य के रूप में प्रयुक्त किए जाने के परिणाम पर एक मूल्याँकन :**

ABSTRACT

The major impediment to the culture of penaeid shrimp in captivity is the inability to obtain ovarian maturation and spawning. The present thesis evaluates the reproductive performance of unablated *Penaeus semisulcatus* (green tiger shrimp), fed the intertidal oligochaete *Pontodrilus bermudensis* as a dietary supplement. The reproductive performance of the test group of *P. semisulcatus* fed *P. bermudensis* as dietary supplement was compared to that of the control group, fed squid and clam alone. Significant improvement was observed in the reproductive performance of test group compared to that of the control group. Maximum maturation response was observed when the worm component formed about 9% of total feed. The biochemical composition and fatty acid profile of *P. bermudensis* was analysed. Among the three growth stages comprising the population of *P. bermudensis*, the nonclitellates were found to have the most suitable biochemical composition for feeding the broodstock. The fatty acid profile of *P. bermudensis* revealed a preponderance of n6 fatty acids especially C20:4 n6, suggesting a possible role of prostaglandins in shrimp maturation and spawning. Experiments were conducted to develop a viable long-term method to culture the oligochaete in land based culture units. Of the three culture systems tried, *P. bermudensis* was found to grow well in wooden boxes with seashore sand as media with cowdung and seaweed or cow dung and leaf litter as organic amendments. A multi unit vermiculture system with periodic harvesting and loading of organic matter is proposed as part of shrimp maturation systems with minimal investment to facilitate continuous supply of worms.

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In the global market penaeid shrimps enjoy prime importance as the most economically important marine product. Because of its unique taste and universal appeal, demand for penaeid shrimps is ever increasing from both export and domestic markets. Diminishing yields from the over exploited natural fishing grounds coupled with the increasing market value resulted in an explosive growth of marine shrimp culture in many countries. In this context, production of large quantities of quality shrimp seed in hatcheries has become imperative. Availability of ripe spawners of the desired species at the proper time is a major pre-requisite in efficient planning of hatchery operations. Except in Japan, where there is an organized industry for the capture and marketing of live shrimps, in all other countries where there is no such trade, getting spawners from the wild by trawling is expensive and uncertain. Because of this induced maturation of captive brood stock received the highest priority among the research works in penaeid shrimp culture. Research works conducted in many parts of the world on shrimp maturation and reproduction have been reviewed by Muthu and Laxminarayana (1982), Primavera (1985), Chamberlain (1988), Harrison (1990), Bray and Lawrence (1992) and Browdy (1992). With the report of Panouse (1943) on precocious ovarian development and egg deposition in eye stalk ablated shrimp, most researches in the induced maturation of penaeids were concentrated on unilateral and bilateral eye stalk ablation techniques. Presently the global scientific community is engaged in the refinement of techniques for induced maturation of commercially important shrimp species, over the already available eyestalk ablation method. In spite of the technological

advancements so far, gaps in basic understanding on a number of areas remain, where further research works will be highly desirable for the optimization of commercial seed production.

The green tiger shrimp *Penaeus semisulcatus* de Haan 1844is a strictly marine species offering great potential for developing aquaculture technology, but is presently remaining neglected for the purpose. The observations by Liao and Huang (1972); Samocha and Lewinsohn (1977); Samocha (1980) and Tseng and Cheng (1981) in Israel showed that *P. semisulcatus* is highly tolerant to environmental fluctuations, grows well in ponds and hence is a promising species for aquaculture purpose. In India *P. semisulcatus* supports a lucrative capture fishery along the Southeast Coast, especially the Tamil Nadu Coast. Due to its hardiness and local availability *P. semisulcatus* shows great potential for sea farming (Maheswarudu *et al.*, 1995).

The present research program was planned with the objective of evaluating the reproductive performance of *P. semisulcatus* brood stock fed the intertidal oligochaete *Pontodrilus bermudensis* Beddard 1895 as a dietary supplement. Apart from high mortality rates, reduction in spawn size and quality with successive spawns leading to increased expenses is a major draw back of eyestalk ablation technique. The present study was aimed on obtaining repeated spawning of unablated *P. semisulcatus* by manipulating brood stock feed. Brood stock diet is reported as a key factor in proper ovarian development, spawning and larval viability. Natural diets (fresh frozen marine organisms) have been found to influence reproductive performance in penaeid shrimps.

The results of the entire research programme are embodied in the present thesis. The general introduction to the study on looks the present status of our knowledge on the subject and the importance of the present investigation.

Subsequent to the general introduction, various aspects of the present investigation are covered in three chapters. Each chapter has an introduction in the beginning, followed by materials and methods, results and discussion. Introduction of each chapter highlights the significance of the particular aspect of study, covering a review of pertaining literature. In the materials and methods, the methodology used, as well as the statistical procedures applied for the data analysis are described. The data obtained are presented in the results, with necessary graphs and tables. Each chapter is concluded with a brief discussion about the salient findings of the study.

Chapter one pertains to the induced maturation study on *P. semisulcatus* brood stock. The reproductive performance of the control group fed standard diet (squid, clam) and the test group fed experimental diet (intertidal oligochaetes and standard diet) was compared and the influence of the intertidal oligochaete *P. bermudensis* on the maturation and spawning of *P. semisulcatus* was evaluated.

In the second chapter detailed biochemical analysis of the dietary supplement, *P.bermudensis* is discussed. The percentage of moisture, protein, lipid, carbohydrate, carotenoid and ash content were estimated for different stages in the life cycle of the intertidal oligochaete. Considering the importance of lipids, especially fatty acids in the maturation of penaeid

shrimps, gas chromatographic analysis of the fatty acid profile of the oligochaete was also carried out, so that a possible relation between the successful inducement of maturation in *P. semisulcatus* and the lipid content of diet, the oligochaete, can be found.

Chapter three deals with the culture of the intertidal oligochaete, *P.bermudensis*. After establishing the essentiality of the worm as a dietary supplement in influencing the reproductive performance of *P. semisulcatus,* it is imperative to ensure its usage in commercial shrimp hatcheries. A viable long-term method at minimal expenditure for the mass culture of *P.bermudensis* with intermittent harvesting is developed.

Subsequent to these chapters is the summary and conclusions, which summarizes the findings of the entire study and the conclusion drawn there of.

The references cited in the entire thesis are presented at the end.

GENERAL INTRODUCTION

Shrimp is the principal seafood commodity traded and accounts for about 20% of the total value of the World's fish trade. The increasing demand for penaeid shrimp from domestic as well as export markets and decreasing catches from natural fishing grounds, directs shrimp farming a highly profitable industry. The most important pre-requisite of successful shrimp culture is the availability of quality seeds from hatcheries. The ability to obtain complete controlled maturation and predictable spawning of viable eggs for timely production of high quality larvae is still a major problem for the shrimp culture industry. Important strides have been made towards the ultimate goal of domestication of penaeid shrimp by the work of various researchers but many gaps are still remaining to be investigated.

Timely availability of gravid female shrimps is the major problem in maturation systems. Dependence on wild gravid females for nauplii production is associated with problems like inconsistent availability, possible negative impacts on fisheries etc. The most popular method for inducing maturation in captive penaeid shrimp is unilateral eyestalk ablation. But this method has its own drawbacks like high mortality rate among brood stock, decreasing spawn size and quality with successive spawns etc. (Emmerson, 1980; Primavera *et al.,* 1982; Poernomo and Hamami, 1983; Lin and Ting, 1986; Choy, 1987; and Makinouchi and Honculada-Primavera, 1987). Because of these disadvantages the eyestalk ablated adults are used once or twice for spawning in commercial hatcheries, leading not only to the sacrifice of a number of spawners from wild but also to increased costs.

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Experiments on control reproduction for the mass production of seed of penaeid shrimps by nutrition without eyestalk ablation are quite limited. Nutrition in relation to reproduction of penaeids is profoundly important and the success of reproduction is closely related to nutrient ingestion accompanying ovarian development. Though proper nutrition is critical to captive breeding, dietary needs of breeders are not well defined either in terms of nutrient requirements, practical ingredients for compounded diets or in terms of the most appropriate fresh food organisms (Bray and Lawrence, 1992). Fresh feed has been found to be superior to pelleted diet for induction of ovarian development in terms of fecundity and larval quality. The choice of feed for brood stock in captivity depends on local availability, preference and reproductive performance of breeders. Observations by various researchers emphasized the importance of nutrition in penaeid reproduction (Middleditch *et al.*, 1979; 1980a and b; Chamberlain and Lawrence, 1981a; Gomez and Arellano, 1987; Cahu *et al.*, 1986 and 1987; Croz *et al.*, 1988; Bray *et al.*, 1989 and 1990; Browdy *et al.*, 1990; Galgani *et al.*, 1989a and b; Kanazawa, 1990; and Lytle *et al.*, 1990).

Reviewing the reproduction of penaeids in captivity, Browdy and Lawrence (1992), outlined the general characteristics of brood stock diets presently in use. In addition to high levels of marine animal source protein (45-65%), lipids, especially fatty acids, play a very important role in inducing maturation and spawning. The dietary lipid requirement of penaeid brood stock is considered to be very high owing to various factors like, the rapid rate of ovarian tissue synthesis (ovaries account for about 10% or more of female weight), accelerated rate of ovarian development with eyestalk

ablation and limited storage function of hepatopancreas, the large lipid content in ovary tissue and limited ability of shrimp to synthesize predominant polyunsaturated fatty acids in ovaries, the inability of shrimp to synthesize sterols and high phospholipid requirement. A major aspect of lipid metabolism is the dietary level of fatty acids. The long carbon chain polyunsaturated fatty acids (PUFA), linoleic (C18: 2n6), linolenic (C18: 3n3), arachidonic (C20: 4n6), eicosapentaenoic (C20: 5n3) and docosahexaenoic (C22: 6n3), were shown to be essential for growth, reproduction and egg and larval development in penaeids by various researchers. Dietary sources of these fatty acids are required, as endogenous synthesis by shrimp is either limited or absent (Kanazawa and Teshima, 1977 and 1981; Kanazawa *et al.,* 1977; 1979 a, b, c, d, e and f; Deshimaru *et al.,* 1979; Bottino *et al.,* 1980; Kayama *et al.,* 1980; Lilly and Bottino, 1981; Dall *et al.,* 1991; Bray and Lawrence, 1992; Middleditch *et al.,* 1980 a; Teshima and Kanazawa, 1983; Teshima *et al.,* 1989; Xu *et al.,* 1994 b; Millamena *et al.,* 1986; Magarelli Jr. 1981; Cahu *et al.,* 1986 and 1987; Millamena, 1989; Lytle *et al.,* 1990; and Croz *et al.,* 1988).

Marine annelids belonging to Polychaeta have been reported as successful shrimp maturation dietary supplement. Improved reproductive performance was observed with the addition of *Glycera dibranchiata* to other fresh feed components or pellets in *P. vannamei* (Gomez and Arellano, 1987; and Ogle, 1988) and *P. setiferus* (Middleditch *et al.,* 1980 b; Chamberlain and Lawrence, 1981a; and Brown *et al.,* 1979); of *Americonuphis reesei* in *P. vannamei* and *P. stylirostris* (Croz *et al.,* 1988); and of *Neries diversicolor* in *P. kerathurus* (Luis and Ponte, 1993). Many species of oligochaetes are used as fishing bait and food for cultured

fishes and invertebrates. Earthworms form the natural feed for many amphibians (Smith, 1951; Lescure, 1966; Lopez, 1979; and Catling and Freedman, 1980) and earthworm meal has been reported to be a better source of protein than conventional protein meals. Stafford and Tacon (1984) reported that the chemical and nutritional characteristics of various species of earthworms were similar to those of fishmeal; and amino acid and polyunsaturated fatty acid profiles of earthworms were particularly suitable for use in commercial fish production. Intertidal oligochaetes from Mandapam coast of Tamil Nadu were observed to improve the reproductive performance of unablated *P. indicus* (Maheswarudu *et al.*, 1996).

The present study evaluates the reproductive performance of unablated *Penaeus semisulcatus,* fed the intertidal oligochaete *P. bermudensis* as a dietary supplement. *P. semisulcatus,* the green tiger shrimp, is an important species supporting the commercial shrimp fisheries of the Southeast coast of India. *P. semisulcatus* was reported to be a highly tolerant species to environmental fluctuations and was found to grow well in ponds (Liao and Huang, 1972; Samocha and Lewinsohn, 1977; Samocha, 1980; and Tseng and Cheng, 1981). Maheswarudu *et al.* (1995), projected *P. semisulcatus* as a potential species for commercial aquaculture along Tamil Nadu coast.

The intertidal oligochaete, *Pontodrilus bermudensis* has a worldwide distribution in the tropical, subtropical and warm tropical regions of Atlantic, Pacific and Indian Ocean (Subba Rao and Ganapati, 1975). In India, the worm has been reported from Chilka Lake, intertidal regions of Pamban, Port Blair, Laccadives, Maldives, Kovalam, Port Okha,

Elephanta and estuarine regions of Visakhapatnam Harbour (Beddard, 1903; Stephenson, 1914; 1915a; 1916 and 1930; Aiyar, 1929; Gates, 1936; Menon and Sareen, 1967; and Subba Rao and Ganapati, 1972; 1974 and 1975).

In the present study the reproductive performance of the test group of *P. semisulcatus* fed *P. bermudensis* as dietary supplement was compared to that of the control group, fed squid and clam alone. Given the importance of proper nutrition in shrimp maturation and spawning and the suggested involvement of dietary fatty acids (Middleditch *et al.,* 1979), the biochemical composition and fatty acid profile of *P. bermudensis* was analyzed to search for possible relations of cause and effect. Attempts were also made to develop a viable long-term method to culture the oligochaete in land based culture units, so that its availability in hatcheries can be ensured. Different culture systems and various media with different organic amendments were tried.

CHAPTER-1

COMPARISON OF THE REPRODUCTIVE PERFORMANCE OF *PENAEUS SEMISULCATUS* FED STANDARD DIET AND EXPERIMENTAL DIET

INTRODUCTION

The increasing global interest in shrimp culture demands large-scale production of quality seed from hatcheries. Successful hatchery operation requires a reliable source of ripe female shrimp of the desired species at the proper time. Gravid females can be procured from three sources: a) catching from the wild in ripe condition, b) taking from culture ponds in a gravid condition, and c) obtaining from either wild or culture ponds in a non-gravid condition and then induce to mature in the hatchery. Most of the commercial hatcheries today rely on the first source. Unfortunately such collections lead to negative impacts on capture fisheries, inconsistent availability, increased expenses, limited potential for production of certain indigenous stocks, endemic disease problems and lack of potential for genetic selection. The second source is currently limited to only a few shrimp species and also in inconsistent numbers to meet the demand from industries for seed. The third source is the alternative to ensure continuous hatchery operation, but its application on a commercial scale is presently impeded by the lack of effective techniques.

Control of gonadal maturation is a major problem while developing commercial aquaculture programmes for most decapod crustaceans

(Yano, 1992). A well recognized and most successful technique for inducing maturation in captivity is the removal of eyestalk that contain the X-organ–Sinus gland complex, which produces and stores gonad inhibiting hormones (Adiyodi and Adiyodi, 1970). Ever since Panouse, (1943), demonstrated for the first time in his classical experiment on *Leander serratus*, that eyestalk ablation during sexual inactivity led to rapid increase in ovarian size and precocious egg deposition, eyestalk ablation has been a research tool for exploring the hormonal mechanisms in various crustaceans, such as *Pandalus kessleri* (Aoto and Nishida, 1956), *Carcinus maenas* (Demeusy, 1967), *Scylla serrata* (Rangnekar and Deshmukh, 1968), *Crangon crangon* (Bomirsky and Klek, 1974), *Baratelphusa cunicularis* (Nagabhushanam and Diwan,1974), *Panulirus argus* (Quackenbush and Herrnkind,1983), *Uca pugilator* (Quackenbush and Keeley,1988) etc. Idyll (1971) and Caillouet (1973) in the USA applied this method in penaeid shrimp culture during early 1970's, on *Penaeus duorarum*. This was followed by the application of bilateral and unilateral eyestalk ablation techniques on various species of penaeid shrimps by researchers from different parts of the world. Bilateral eyestalk ablation, although leading to rapid ovarian growth, did not result in spawning as the ova got reabsorbed without being released from the ovary. (Caillouet, 1973; Duronslet *et al.*, 1975; AQUACOP, 1975; Wear and Santiago, 1976). At the same time unilateral eyestalk ablation was found to result in successful ovarian maturation and subsequent spawning (Arnstein and Beard, 1975). Over the years a lot of research has been done by applying unilateral eyestalk ablation on various penaeid species under various conditions and some of the contributions worth mentioning are by, Alikunhi *et al.* (1975), Wear and Santiago (1976), AQUACOP (1977a and 1979), Primavera (1978a and b; and1982), Primavera *et al.* (1978 and 1982),

Primavera and Yap (1979), Rodriquez (1979), Lumare (1979), Muthu and Laxminarayana (1979 and 1980), Emmerson (1980), Kulkarni and Nagabhushanam (1980), Pudadera and Primavera (1981), Yano(1984), Chamberlain and Gervais (1984), Browdy and Samocha (1985a and b), Browdy *et al.*(1986), Choy (1987), Makinouchi and Honculada – Primavera (1987), Wyban *et al.*(1987), Mohammed and Diwan (1991), Cardona and Capo (1992) and Rao *et al.*(1993).

Though eyestalk ablation is widely used at present for successful ovarian maturation and spawning of captive shrimp, it was found to increase female mortality, (Emmerson, 1980; Primavera *et al.*, 1982; Poernomo and Hamami, 1983; and Makinouchi and Honculada-Primavera, 1987) mainly due to bacterial infection in the wound. Eyestalk ablation also deteriorated female condition (Emmerson, 1980), lowered hatch rate of eggs and decreased larval viability with repeated spawning (Emmerson, 1980; Lin and Ting, 1986; and Choy, 1987). Accounting the negative impacts of eyestalk ablation on brood stock management, attempts were made to induce maturation in shrimp without eyestalk ablation. Unablated penaeids were reported to mature and spawn naturally in land based maturation tanks under controlled environmental conditions by Moore *et al.* (1974), AQUACOP (1975; 1977b; and 1979), Beard *et al.* (1977), Laubier–Bonichon (1978), Caubere *et al.* (1979), Emmerson (1983), Emmerson *et al.* (1983), Muthu *et al.* (1984 and 1986), Cripe (1994) and Maheswarudu *et al.* (1996).

Most of the initial works on maturation of penaeids under artificial conditions have concentrated on eyestalk ablation and related hormonal changes or on the influence of environmental factors. Comparatively, only very few controlled experiments have been carried out on the nutritional

requirements of female brood stock (Primavera, 1985). In penaeids successful reproduction is closely related to nutrient ingestion accompanying ovarian development. In spite of the critical importance of proper nutrition to captive breeding, dietary needs of penaeid brood stock are poorly defined in terms of nutrient requirements, practical ingredients for compounded diets and in terms of the most appropriate fresh food organisms (Bray and Lawrence, 1992). Usually the brood stock diet consists of a combination of fresh food organisms, supplemented by a small portion of dry prepared feed. The importance of fresh natural feed in maturation is an established fact. Commonly used natural diets are fresh or fresh-frozen marine organisms like squid, mussel, clam, shrimp, brine shrimp and polychaete worms. Additionally, fish, shark, mysids, troca, cockles, krill, crab and marine algae have been reported as breeding diets (AQUACOP, 1977a; Emmerson, 1980; Chamberlain and Lawrence, 1981 a and b; Emmerson *et al.*, 1983; Gomez and Arellano, 1987; Bray and Lawrence, 1988 and 1992; Bray *et al.*, 1989 and 1990; Browdy, 1989; and Galgani *et al.*, 1989a and b.) Attempts to replace fresh natural diets completely with prepared dry feed have so far been unsuccessful. Dry feeds alone were unable to elicit the same maturation response as combinations of fresh feeds (Bray *et al.*, 1990; Redon and San Feliu, 1993b; and Sangpradub *et al.*, 1994). Prepared dry feeds are frequently used as supplements comprising 20-25% of a maturation diet. Emmerson (1983) and Emmerson *et al.* (1983) used higher percentages of dry feeds in their studies.

Penaeus semisulcatus de Haan, the green tiger shrimp, is an important species supporting the commercial shrimp fishery along the South East

coast of India. It grows well in ponds and is highly tolerant to environmental fluctuations (Liao and Huang, 1972; Samocha and Lewinsohn, 1977; Samocha, 1980; Tseng and Cheng, 1981; and Maheswarudu *et al.* 1997). Maheswarudu *et al.* (1995) suggested *P. semisulcatus* as a potential species for commercial culture along Tamil Nadu coast. Its hardiness, local availability and disease resistance projects *P. semisulcatus* as an alternate species to Indian shrimp culture industry, which is suffering from disease out breaks. Thomas (1974), conducted investigations on the reproduction, fecundity and sex ratio of *Penaeus semisulcatus* from Mandapam waters of Tamil Nadu coast. Browdy (1989) discussed the reproductive biology of *P. semisulcatus* from Israeli waters. Bose (1995) studied the reproductive biology of this species from Gulf of Mannar and Palk Bay along South East coast of India.

In India, experiments on the nutritional requirements of shrimp brood stock in captivity are quite limited. Muthu and Laxminarayana (1977) reported maturation and spawning in ablated *P. indicus* fed fresh clam and live mysids. Maheswarudu *et al.* (1996) obtained prolonged repetitive spawning in unablated *P. indicus* fed intertidal oligochaetes, clam and squid as maturation diet. The present chapter evaluates the reproductive performance of *P. semisulcatus* fed the intertidal oligochaete *P. bermudensis* as a dietary supplement. Maturation experiments were carried out in rematuration systems and reproductive performance was compared between control group fed standard diet and test group fed standard diet plus oligochaete worms.

MATERIALS AND METHODS

Collection, transportation and acclimatization of brood stock:

Healthy breeders of *P.semisulcatus* de Haan were collected by trawl net operations in the Gulf of Mannar and Palk Bay during June 1998. Animals were soon transported in aerated seawater to the backyard hatchery at Mandapam Regional Centre of Central Marine Fisheries Research Institute, Mandapam and were acclimated for 10-15 days before beginning the experiment.

Experimental setup:

i. Rematuration tanks: Two circular fibreglass tanks of five-ton capacity each were used for maturation experiments. Each tank had a sand bed (about 10cm height) filter laid on a perforated aluminum false bottom that stood at about 15cm height over the entire bottom. Water after filtration through the sand bed got collected under the false bottom and was air lifted to the surface through four PVC tubes (4cm dia) that were fixed inwardly in the peripheral region of the tank at equal distance. The rate of water recirculation in the maturation tank was fifteen times per day. The tanks were covered with lids made of aluminum sheet with wooden frames to cut the light intensity. Inner sides of the tanks were smooth to avoid injury to the shrimps due to abrasion.

ii. Spawning tanks: Cylindro-conical fiberglass tanks of 150L capacity with bottom- drain facility were used for spawning. Aeration with a single air stone was provided in the tank.

iii. Aeration: Continuous aeration was provided with an air compressor run by 5H.P. electrical motor.

iv. Source of seawater: Seawater that pumped from Gulf of Mannar was allowed to settle for a night in the settlement tanks and then passed through sand filter to the storage tank. Then water was drawn by a one H.P. monoblock pump set to an overhead tank for day-to-day activities. From the overhead tank water supply was provided to maturation tanks and spawning tanks through PVC pipelines. Water was filtered through a 5μ mesh cloth lined bag before use.

Experimental procedure:

i. Brood stock preparation: A total of fourteen animals- seven males and seven females- were stocked in each tank. While stocking care was taken to balance the shrimp size of both the tanks. At the start of the experiment, shrimp weights and lengths were recorded. Each shrimp was double marked for later individual identification, to establish moult time and to document ovarian maturation. A double marking consists of numbered plastic tags fastened around the ocular peduncle of right eye (Plate 1.2 and 1.3) and clipping the distal portion of uropod/antennae (Table1.1). The coded cuts of uropods/ antennae allowed identification of moults as well as rapid identification of individuals in the tanks while sourcing spawners. These marks were renewed whenever cut portions regenerated. The ring tag around the eyestalk remained as a permanent identification. Separate colours were used for male and female shrimps of each tank.

ii. Feeding: Diet consisted of combination of fresh feed items. The control group of shrimps was fed with the standard diet and the test group was fed with the experimental diet. The standard diet consisted of squid (*Loligo*

Plate 1.1. Rematuration system described in the present study

Plate 1.2. Test group females of *P. semisulcatus*

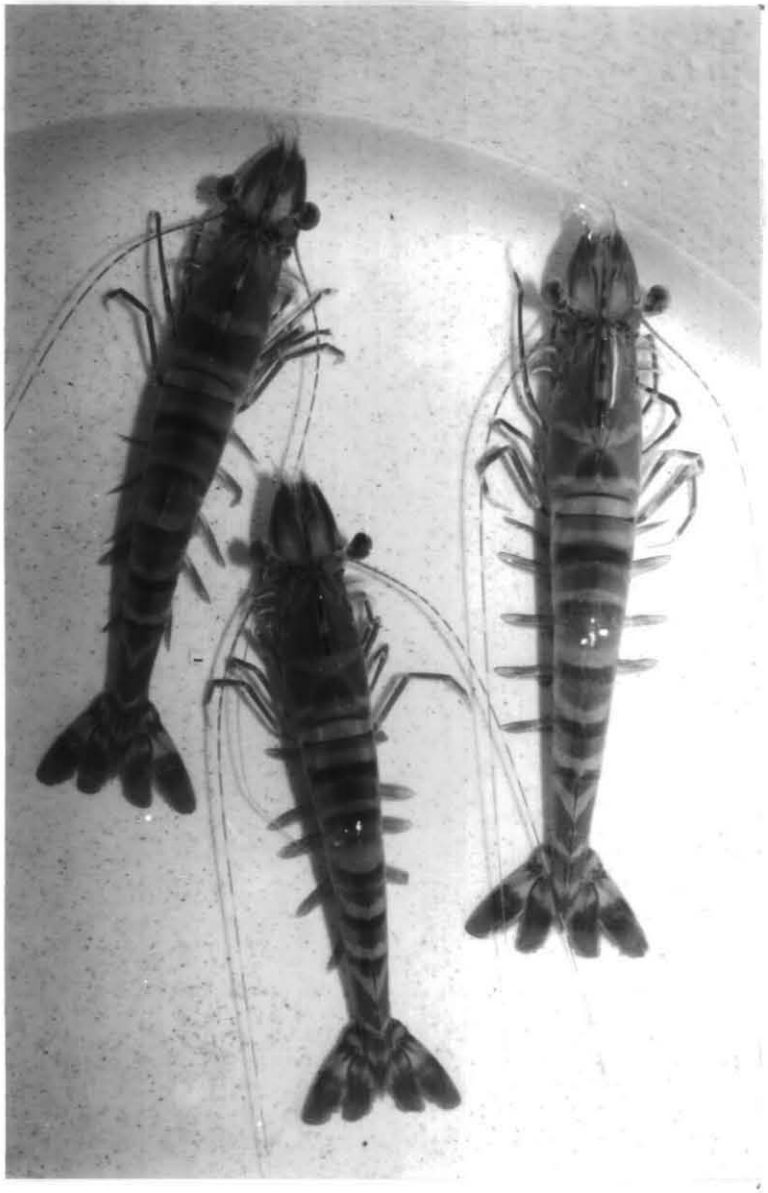


Plate 1.3. Control group females of *P. semisulcatus*

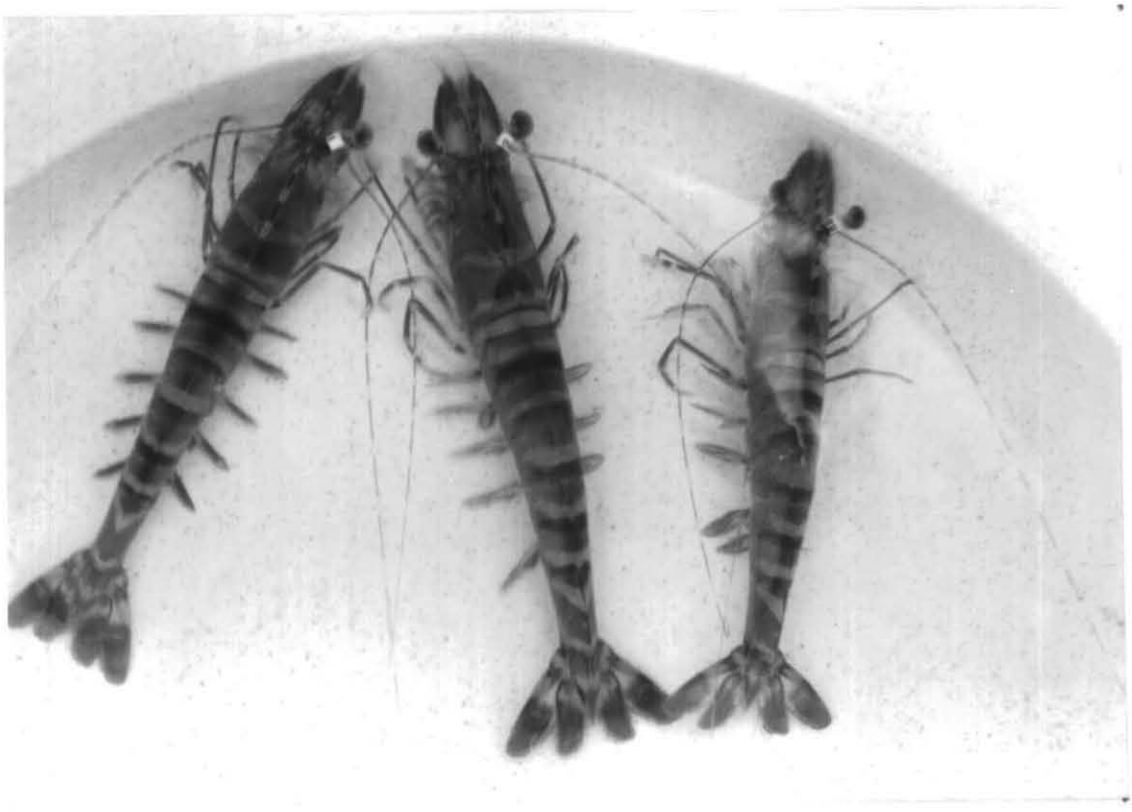


Table 1.1: Coded cut marks used for individual identification of shrimps in each tank.

Shrimp number	Identification cut mark
1	First uropod from right
2	Second uropod from right
3	Third uropod from right
4	Fourth uropod from right
5	First and second uropod from right
6	Third and fourth uropod from right
7	First antenna from right

sp.) and clams (*Anadara* sp.). The experimental diet was constituted by standard diet plus a certain amount of the intertidal oligochaete, *P. bermudensis*. The total quantity of feed given to both the groups was same initially. During acclimatization both the groups were fed with standard diet.

At the beginning of the experiment, shrimp were fed twice daily, at 09.00hrs and 16.00hrs, but *P. semisulcatus*, being nocturnal and burrowing, were found to accept the feed only at night, so that later feeding was restricted to 16.00hrs only. In maturation experiments the rule is to feed the brood stock *ad libitum*, so that little feed is always left in the tank by next morning. Initially shrimps in both the tanks were fed 10% of their total biomass per day, which was periodically increased up to 12% of total biomass in the control tank and 18% of total biomass in the test tank depending on the demand. Initial percentage of clams and squid in the standard diet was 3 and 7 respectively, and clams, squid and worms in the test diet were 3, 4 and 3 respectively. The gradual increase in total feed and corresponding increase in percentage of ingredients as per the demand and preference of breeders are given in Table 1.2. The control group females always preferred clams to squid, whereas the test group breeders preferred worms first, clams second and the squid lastly. Squid was always found as the excess feed in both the tanks. Therefore the percentage of squid was decreased gradually and the percentage of worms increased in the test diet. The percentage of clams in the control and test diets remained the same always.

Table 1.2: Feeding strategy during the experimental period

Day	Control			Test			
	%Total biomass	%Clam	%Squid	%Total biomass	%Clam	%Squid	%Worm
0	10	3	7	10	3	4	3
16	10	4	6	10	4	2	4
47	12	5	7	12	5	2	5
68	12	5	7	14	5	2	7
74	12	5	7	16	5	2	9
124	-	-	-	18	5	2	11

iii) Maintenance of brood stock: During the experimental period utmost care was taken to ensure proper functioning of water recirculation system and in the removal of excess feed and feacal matter, which may lead to deterioration of water quality and resorption of developing ovaries. Every morning, fresh exoskeletons, if present were recovered from the tanks, identified the shrimp with the help of coded cut marks and recorded. Freshly moulted females were checked for proper spermatophore insemination. Dead prawns were collected and duly recorded. The brood stock were given a 20 minute dip treatment in 100ppm formalin once in every month as prophylaxis

iv) Sourcing of mature females and spawning: Female shrimp were checked for ovarian maturation daily night at 19.00-20.00hrs by a spot light. Females with fully mature ovaries, visible as greenish mass through the dorsal exoskeleton were scooped out gently and transferred, individually, to spawning tanks filled with fresh filtered seawater treated with disodium salt of EDTA (0.1g/100L). Aeration in the spawning tanks was minimized to 4-5 bubbles per second and the tank was covered with a net to prevent jumping ~~out~~ of the spawner. Complete darkness was provided in the hatchery during night hours by switching off the lights. In the following morning (06.00hrs) females in the spawning tanks were checked for spawning by observing the presence of floating protenacious scum released during spawning. Water was microscopically examined for eggs. Spent females were returned to their respective maturation tank. Unspawned females were also returned to the concerned maturation tanks but kept for spawning for another 1-2 nights. Ovaries began reabsorption within three days whether spawning occurred or not. After spawning the female was removed and aeration in the spawning tank was

increased to ensure better movement of eggs and consequent good hatching rate.

v) Estimation of eggs, nauplii and hatch rate: Water in the spawning tank was stirred well to achieve a uniform suspension of eggs. Three 100mL aliquots were drawn from this and counted the number of eggs in each sample. Average egg count of the three aliquots was multiplied by 1500 to get total estimated eggs spawned. The eggs were left in the spawning tanks with aeration and allowed to hatch. Total number of nauplii was determined after 16-18 hours from spawning by counting number of nauplii in three 100mL aliquots drawn from the tank as mentioned above. Hatch rate was estimated by the following formula:

$$\text{Hatch rate} = \frac{\text{Total nauplii}}{\text{Total eggs spawned}} \times 100$$

Date of spawning, total number of eggs spawned and total number of nauplii hatched in each spawning of every female were recorded.

vi)Maintenance of physico-chemical parameters of water in the maturation system: Water temperature and pH of the rematuration tanks were monitored daily. Water temperature was, measured using a graduated mercury thermometer (accuracy up to 0.01°C) at 09.00 hrs and 16.00hrs. pH was determined using an Elico pH meter in the morning hours. Once in three days, the ammonium-N, was determined by the indophenol method, and nitrite–N was determined by sulfanilamide method (Boyd and Tucker, 1992). Dissolved oxygen (DO) and salinity was determined once in five

days. DO was measured by the Winkler's method (Strickland and Parsons, 1972; and Spotte, 1979) and salinity was measured using argentometric method (Strickland and Parsons, 1972).

Statistical analysis:

Students t-Test was performed to compare the reproductive performance of the two groups.

RESULTS

The experiment lasted for a period of 192 days until all the females died. The physico-chemical parameters of water in the maturation tanks during the experimental period are listed in Table 1.3. Water temperature varied from 27.1°C - 31.0°C; pH ranged from 7.09 – 8.6; salinity fluctuated in between 35.2‰ and 36.4‰ and dissolved oxygen ranged from 3.5 mg/l to 5.0 mg/l. Ammonia and nitrite levels in the maturation system are limiting factors for induction of maturation. Total ammonia level ranged from 0.00 to 0.01 and nitrite content varied from 0.002 to 0.05. No significant differences were observed between the water parameters of control and test groups.

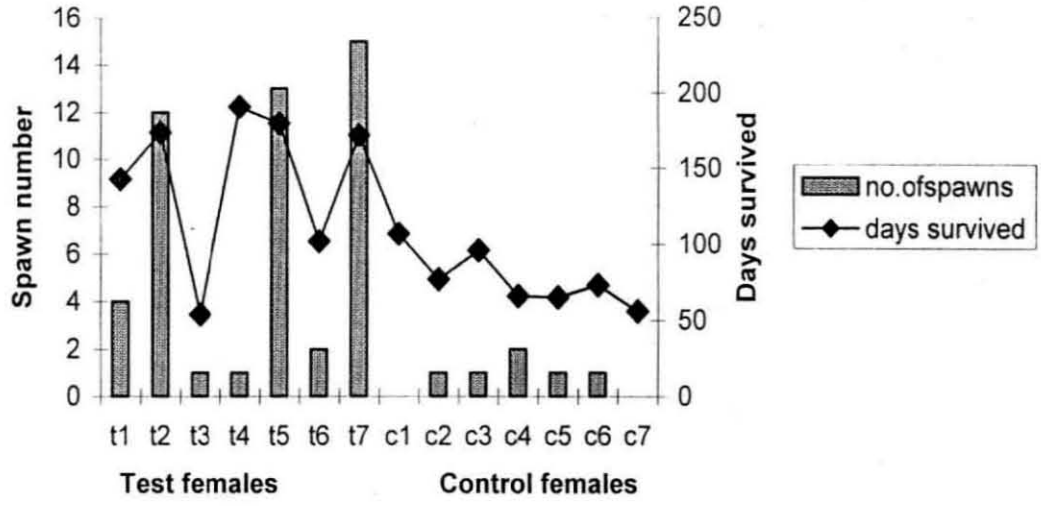
There was significant difference between control and test groups in the number of days shrimp survived in the experimental system (P=0.005). The test animals survived for an average of 152.143 ± 11.705 days, while control animals survived only for an average of 75.214 ± 4.303 days (Fig.1.1).

A total of 191 moults were collected and identified during the experimental period. The number of moult cycles undergone by the test animals was

Table1.3: Physico-chemical parameters of water in the maturation tanks during the experimental period.

Tank	Temparature (°C)				pH		Salinity (‰)		D.O. (mg/l)		Total Ammonia (mg/l)		Nitrite (mg/l)	
	09.00hrs		16.00hrs											
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Control	28.6	27.1-30.1	29.1	27.3-30.6	8.2	7.42-8.49	35.9	35.6-36.4	4.24	3.5-4.9	0.0003	0.00-0.010	0.014	0.002-0.050
Test	28.5	27.1-30.8	29.2	27.3-31.0	8.2	7.09-8.6	35.3	35.2-36.4	4.5	3.6-5.0	0.0003	0.00-0.009	0.012	0.002-0.04

Fig:1.1 Survival and spawning of *P. semisulcatus* females in the experimental system



significantly higher than that of control (P=0.0003), but at the same time there was no significant difference in the duration of moult cycle between the two groups (P=0.1901). Freshly moulted females invariably had stage1 or stage 2 ovaries, except female 6 of test group which moulted with stage 3 ovary and later reabsorbed.

Altogether 71 maturations were recorded among the two groups, of which 54 resulted in spawning. The test group females gave 48 spawnings while the control group spawned only 6 times (P=0.013). The test group produced a total of 54,73,550 eggs and 46,84,800 nauplii in 48 spawnings while the control group produced only 4,12,500 eggs and 3,23,250 nauplii. The test animals averaged 6.86 ± 2.34 spawns per female, producing an average of 7,81,935 ± 3,12,279.23 eggs per female, whereas the control females spawned an average of 0.86 ± 0.26 times producing an average of 58,928.57± 18,607.93 eggs. The average number of nauplii produced per female was also significantly greater in the test group than that of control female (P=0.020). All the females in the test group spawned at least once, while only 71% of the control group spawned at least once. A comparison of reproductive performance of control and test group *P. semisulcatus* is given in Table 1.4.

Spawns from control group were smaller than that of test group (68,250 ± 3,436.93 vs. 92,251.85± 13,074.04, P = 0.010) and a corresponding decrease in the number of nauplii per spawn (P = 0.0168) was also recorded. There was no significant difference in fertility and hatch rate between the two groups (P=0.049 and P=0.053 respectively). The rate of spawning and egg production (number of spawns / number of days survived and total number of eggs produced / number of days survived) was greater in the

Table 1.4: Comparison of reproductive performance of *P. semisulcatus* between control and test group.

Parameter	Control*	Test*	*P*
Average no. of spawns / female	0.86 ± 0.26	6.86 ± 2.34	0.012
Average no. of eggs produced / female	58,928.57 ± 18,607.93	7,81,935 ± 3,12,279.23	0.019
Average no. of nauplii produced / female	46,178.57 ± 15,046.23	6,69,935.71 ± 2,71,750.55	0.020
Average % fertility	59.32 ± 13.42	86.94 ± 3.16	0.049 n.s.†
Average % hatching	55.53 ± 14.53	81.37 ± 3.38	0.053 n.s.†
Average no. of eggs / spawn	68,250 ± 3,436.93	92,251.85 ± 13,074.04	0.010
Average no. of nauplii / spawn	37,929 ± 10,189.09	76,391 ± 12,381.52	0.016
Average rate of spawning for days survived (number of spawns / day)	0.01 ± 0.004	0.05 ± 0.012	0.016

Average rate of egg production for days survived (total eggs / day)	763.89 ± 275.11	5,387.35 ± 1,711.52	0.021
Average rate of moulting for days survived (number of moults / day)	0.060 ± 0.005	0.062 ± 0.003	0.340 n.s.†
Average number of days for induction of first maturity after introduction into the experimental system	66.57 ± 18.53	21.6 ±5.33	0.038
Average number of days from moulting to spawning	11.4 ± 8.63	7.55 ± 3.02	0.046

* Plus or minus (±) values as per standard errors.

† Statistical tests were run with arctangent transformations.

test group than that of control group (P=0.016 and P=0.021 respectively). This difference was facilitated by a significant difference in the time needed for ovarian development, between moulting (ovarian stage 1-2) and spawning, 7.55 ± 3.02 days for test group and 11.4 ± 8.63 days for control group (P=0.046). The shortening of maturation time led to an increase in the number of spawns per moult cycle for test females (Table 1.4). A maximum of four spawns per moult cycle were observed for test group, where as the control group spawned only once in a moult cycle (Fig: 1.2).

There was no significant difference between the first and the later spawns in terms of size and hatchability in the test group. Table 1.5 shows the summary of repeated spawnings performed per moult cycle of test group. The relative frequency of first, second, third and fourth spawning in the moult cycle was 54.17%, 27.08%, 14.58% and 4.17% respectively.

Particulars of the individual reproductive performance of control and test group females are given in Table 1.6 and 1.7 respectively. Female 7, 5 and 2 of the test group gave the maximum number of spawns. Figures 1.3 - 1.9 detail the spawning performance of all the females in the test group.

DISCUSSION

The chapter documents the reproductive performance of female *P.semisulcatus* fed the intertidal oligochaete, *P.bermudensis* as dietary supplement. In the present study, the physico chemical parameters of

Fig.1. 2: Repeated spawns per moult cycle performed by *P. semisulcatus*

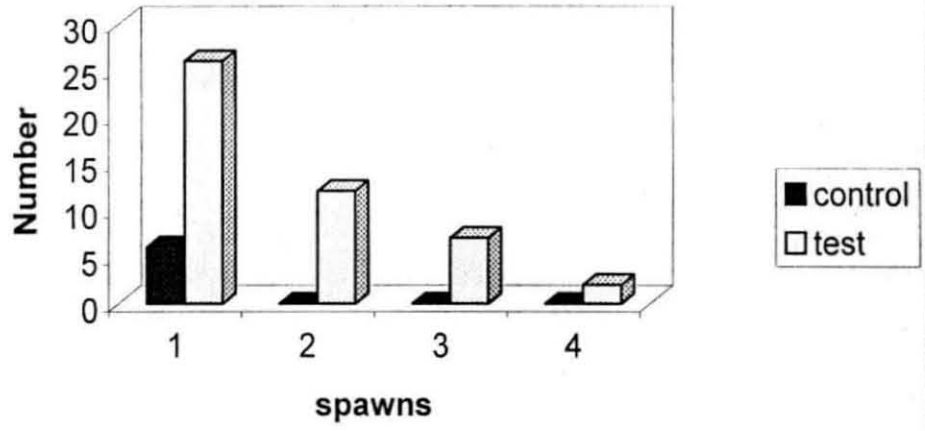


Table 1.5: Synopsis of repeated spawnings per moult cycle of test group *P. semisulcatus*.

Spawn	No.	Frequency %	Mean egg no.	Mean n no.	% Hatch
1	26	54.17	109832.7	94192.31	85.76
2	13	27.08	104338.5	87869.23	84.22
3	7	14.58	134600.0	115071.40	85.49
4	2	4.17	159750.0	144000.00	90.14

Table 1.6: Reproductive performance of control group females

	Spawner number						
Parameter	1	2	3	4	5	6	7
Total length in mm	171	158	167	154	157	165	142
Weight in gm	42	38	40	45	45	50	22
No. of spawning performed	0	1	1	2	1	1	0
No. of moult cycles undergone	7	4	4	4	4	6	3
Range of spawnings performed per moult cycle	--	0-1	0-1	0-1	0-1	0-1	--

Range of duration of moult cycle in days	11-18	18-23	11-17	8-24	12-21	13-15	13-28
Average duration of moult cycle	15.67±0.8	20.67±1.45	15.33±1.20	15.33±4.67	16.00±2.65	14.00±0.45	21.50±6.5
Total no. of eggs spawned	0	60000	82500	142500	66000	61500	0
Total no. of nauplii obtained	0	52500	66000	115500	45000	44250	0
Average no. of eggs / spawn	--	60000	82500	71250	66000	61500	--
Average no. of nauplii / spawn	--	52500	66000	57750	45000	44250	--
Average hatching rate in %	--	87.5	80.0	81.05	68.18	71.95	--

Table 1.7: Reproductive performance of test group females.

Parameter	Spawner number						
	1	2	3	4	5	6	7
Total length in mm	145	169	160	133	143	157	146
Weight in gm	25	50	45	22	25	32	40
No. of spawning performed	4	12	1	1	13	2	15
No. of moult cycles undergone	8	10	4	13	10	7	11
Range of spawnings performed per moult cycle	0-3	0-3	0-1	0-1	0-3	0-1	0-4
Range of duration of moult cycle in days	14-20	12-19	9-15	14-18	13-20	13-18	15-20

Average duration of moult cycle	18.00±0.9	16.30±0.62	12.25±1.31	16.10±0.53	17.11±0.89	16.83±0.83	16.60±0.52
Total no. of eggs spawned	25800	1770500	63000	55500	1253000	186000	1887550
Total no. of nauplii obtained	186000	1530400	57000	375000	1127200	145000	1601200
Average no. of eggs / spawn	64500	147541	63000	55500	96384	93000	125836
Average no. of nauplii / spawn	46500	127533	57000	37500	86707	72750	106746
Average hatching rate in %	72.09	86.44	90.48	67.57	89.96	78.23	84.83

Fig.1.3: Spawning performance of female 1 of test group

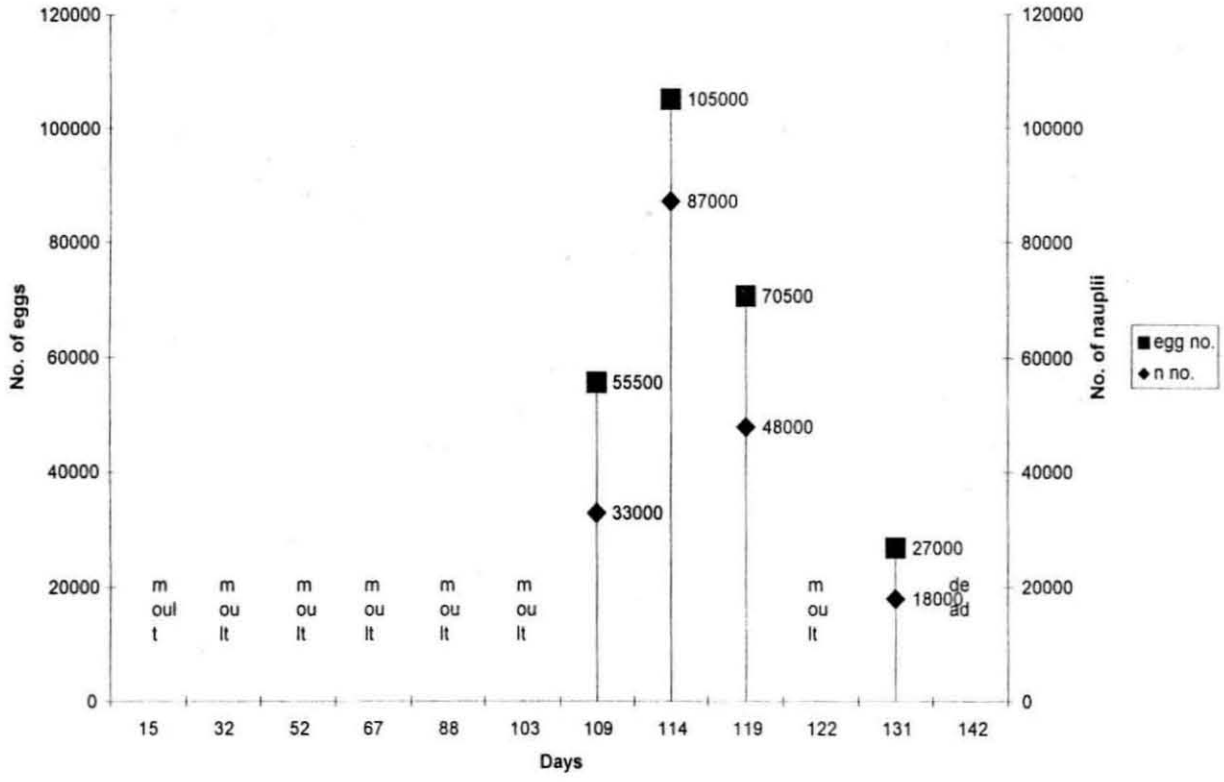


Fig. 1.4: Spawning performance of female 2 of test group

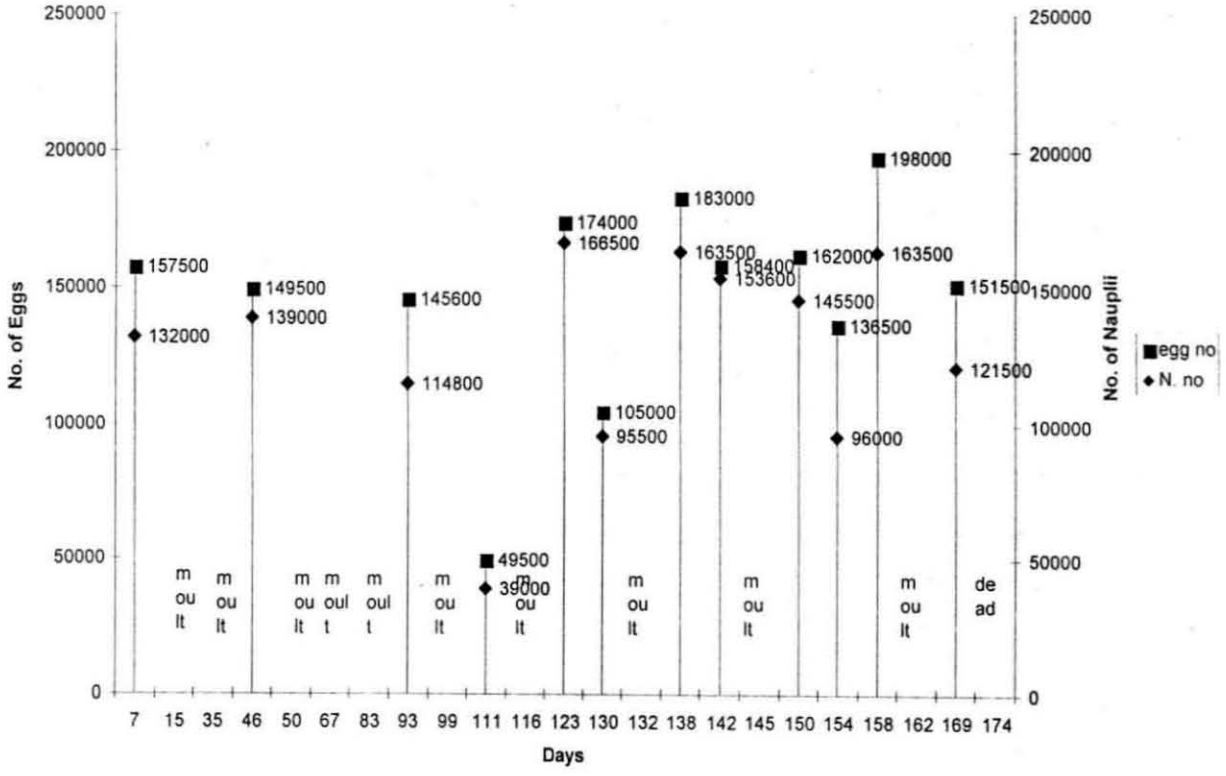


Fig. 1.5: Spawning performance of female 3 of test group

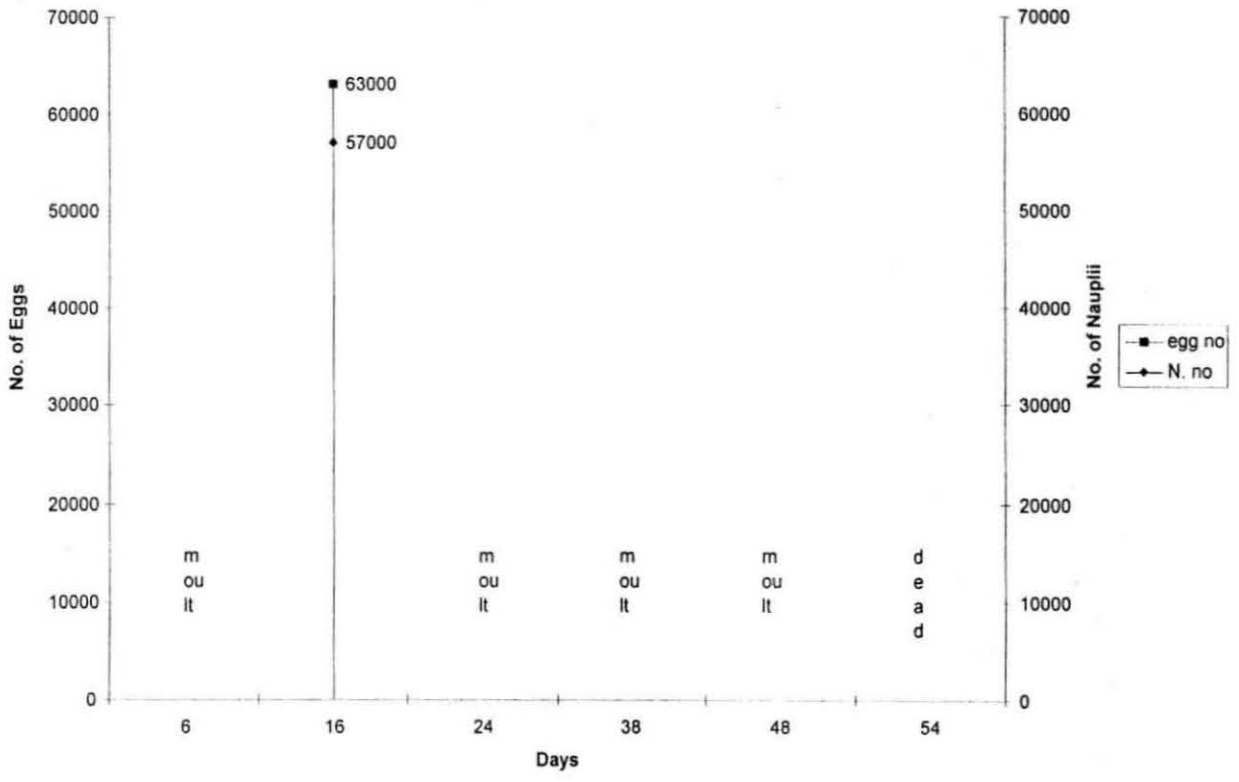


Fig. 1.6: Spawning performance of female 4 of test group

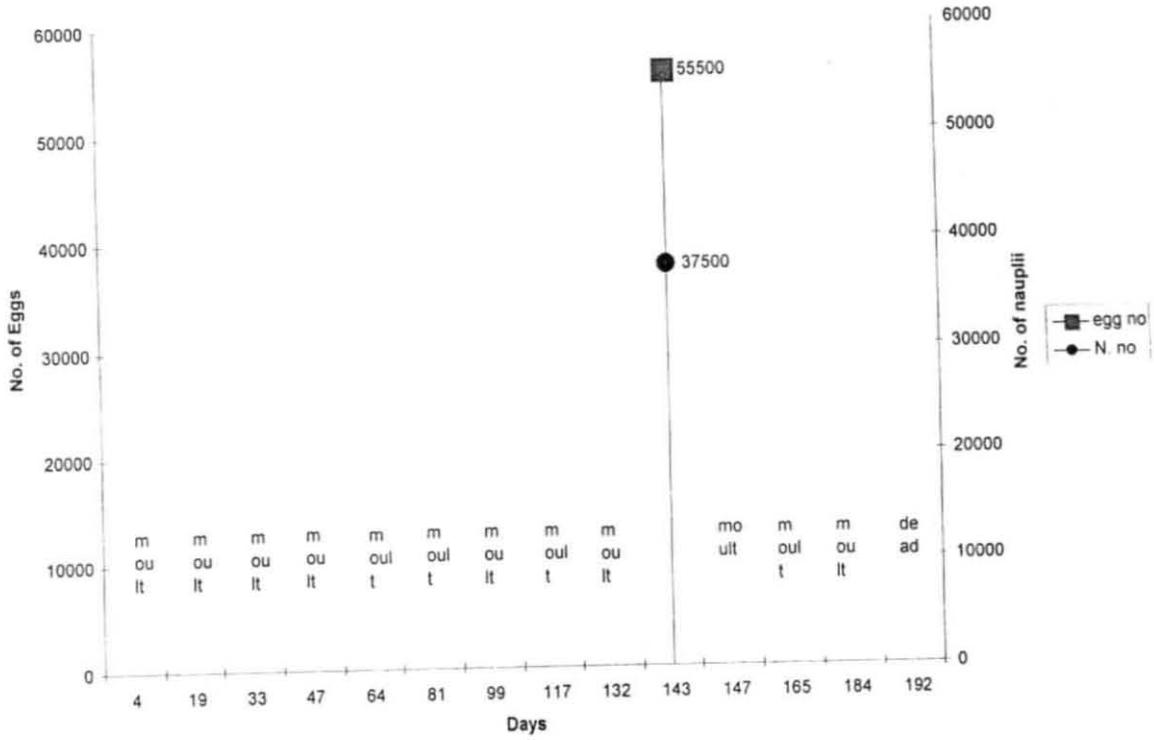


Fig.1.7: Spawning performance of female 5 of test group

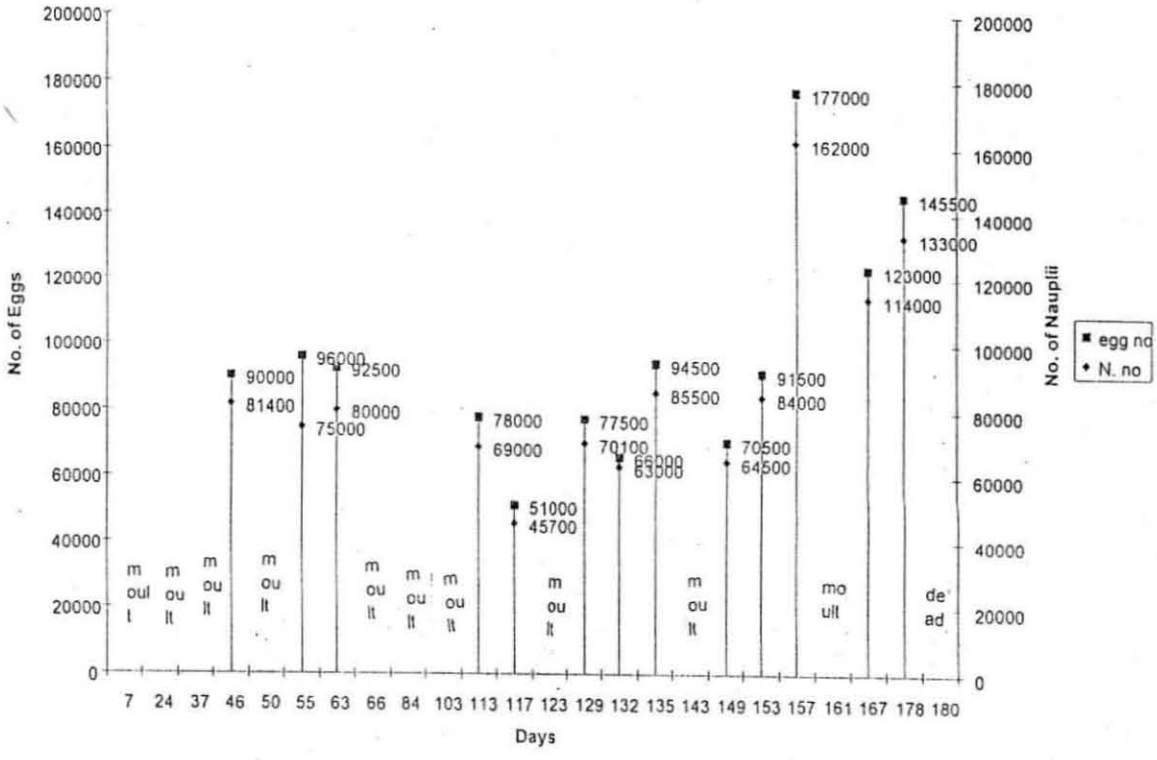


Fig.1.8: Spawning performance of female 6 of test group

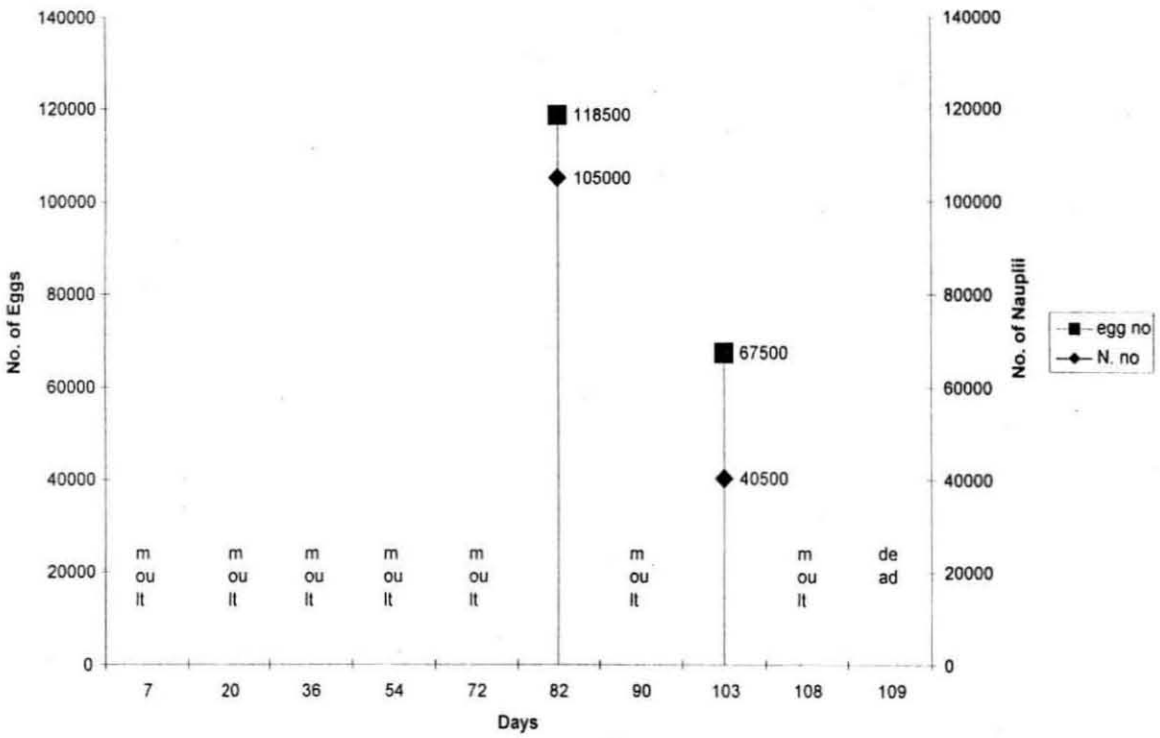
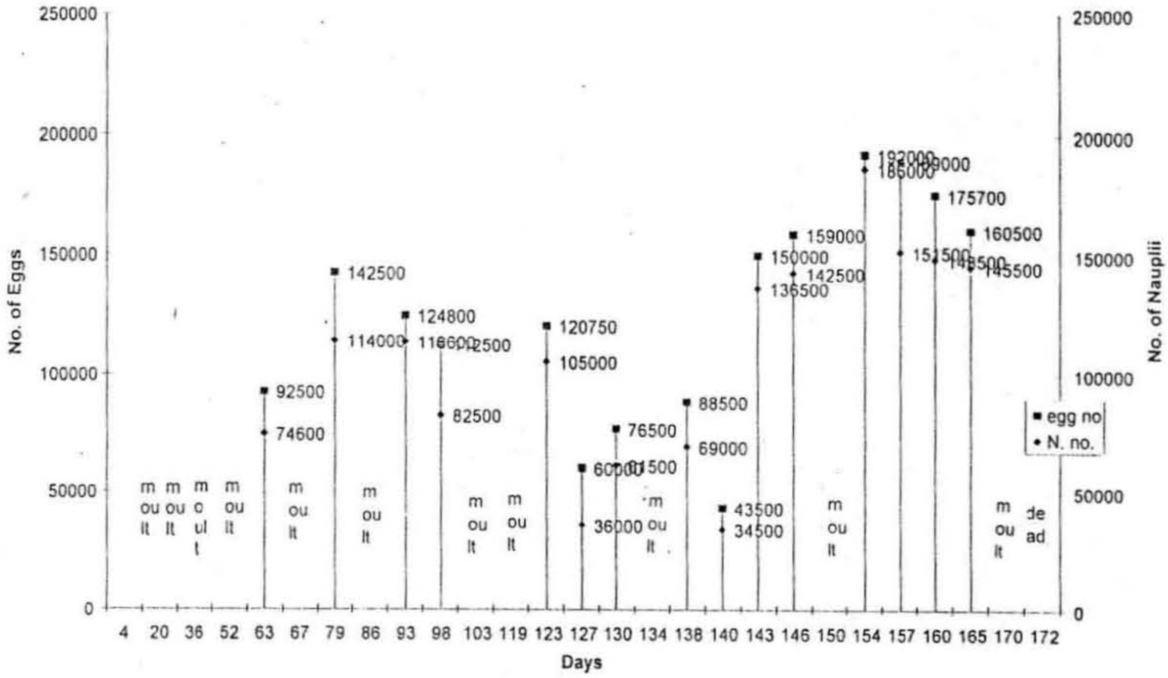


Fig. 1.9: Spawning performance of female7 of test group



both the maturation tanks were similarly maintained. Environmental and system factors other than diet that can affect the reproduction of penaeid shrimp are numerous and are reviewed by Bray and Lawrence (1992). However within each of the maturation tanks such factors can safely be considered constant for both the tanks and therefore are unlikely to have contributed to the observed differences.

Marked difference was observed in the survival of shrimps between the two dietary treatments. In spite of the accidental deaths of two females in the test tank, the total survival of shrimps was longer than that of the control group. Browdy and Samocha (1985a) reported an average survival of about 75 days in captivity for eyestalk ablated and unablated females of *P.semisulcatus*, whereas in the present study the test group females survived for an average of 145 days.

The reproductive capacity of a maturation system depends on the combined performance of both sexes: potency and ability of the males to transfer spermatophores, maturation rate of the females, and degree of fertilization and egg/larval quality. From the present experiment it seems that the effect of the male diet on mating and fertilization success is insignificant, because males fed the standard diet and experimental diet performed equally well. Though the male contribution to reproductive success was not specifically evaluated, it may be assumed that the differences in the reproductive performance were not specifically related to male performance as factors like impotence and inadequate sex ratio were absent in both treatments. The male to female ratio was 1:1 at the time of stocking and the number of males was always adequate during the course of the experiment. {Optional range 1-1.5:1 male: female, Bray and

Lawrence, (1992)}. Also, it is assumed that males with fully developed spermatophores only participated in mating.

At the same time, the present experiment clearly demonstrates the effect of brood stock diet on female reproductive performance. When the standard diet was supplemented by *P. bermudensis*, there was significant improvement in the maturation and fertilization, resulting in a higher number of females spawned and a higher number of nauplii produced per unit spawn.

Proper nutrition has a critical role in captive breeding of penaeids. In nature, an organism utilizes the available nutrients firstly for body metabolism, secondly for growth and then only for reproduction. The organism would not be anticipated to develop ovaries, constituting about 10% of female body weight, unless nutrients are available for metabolism and growth. In captive breeding these nutrients have to be supplied in required amounts so that repetitive spawning and quality seed production is achieved. Dietary factors clearly have been shown to influence percentage of females spawning, percentage hatch, survival and normal physical development of larval stages (Bray and Lawrence, 1992). Though the dietary needs of penaeid shrimp brood stock has not been defined completely, researchers are of the opinion that a combination of different fresh feeds or, fresh feeds and certain percentage of dry feed always give better maturation response than dry feed alone (Bray *et al.*, 1990). In the present study fresh/fresh-frozen feeds alone were used. Redon and San Feliu (1993) pointed out that natural diet gave the greatest percentage of maturation in unablated *P. japonicus*. Sangpradub *et al.* (1994) compared the reproductive performance of three groups of *P.*

monodon brood stock, fed fresh diet, combination of fresh diet and formulated pellet diet and formulated pellet diet only respectively. They found maximum maturation and spawning events for the group fed fresh diet alone, followed by the group fed the combination diet.

The combination of worms, squid and clams (experimental diet) was found to be superior to the combination of squid and clam alone (standard diet). Squid is a good source of protein and is used as a common denominator in maturation diets (Gomez and Arellano, 1987; Galgani *et al.*, 1989 a and b; Bray *et al.*, 1990 and 1992; and Menesveta *et al.*, 1994). Kanazawa (1990) reports that clam meat contains protein and lipid fractions helping in maturation.

Browdy and Samocha (1985a) in their induced maturation experiments in *P. semisulcatus* with eyestalk ablation, observed a significant increase in the number of spawns and corresponding eggs/nauplii per female, for ablated females compared to unablated control shrimps, whereas the spawn size and corresponding number of nauplii, decreased with ablation. In the present study, the average number of spawns per female and average number of eggs and nauplii per spawn were higher for the females fed experimental diet due to absence of eyestalk ablation. Browdy and Samocha (1985a) observed a significant increase in number of spawn and corresponding number of eggs and nauplii per female while the spawn size and number of nauplii decreased with ablation. Hence induction of maturation in penaeid shrimp with proper diet has more advantages than resorting to eyestalk ablation.

The present experiment documents repeated spawning up to four times within one moult cycle for the test group while the control group averaged only single spawn within a moult cycle (Fig. 1.2). Emmerson (1980) reported up to four and up to three evacuations per moult cycle for ablated and unablated *P. indicus* females, respectively but observed a decrease in hatch success (number of nauplii) with successive spawn in a moult cycle. Browdy and Samocha (1985a) demonstrated repeated spawning up to four times per moult cycle for ablated and up to two per moult cycle for nonablated *P. semisulcatus* females. They observed a significant reduction in average spawn size after the first spawn but found that the percent fertilization was similar for all the spawns. Among the test group females, in the present work, no reduction in average number of eggs and hatch rate for repeated spawnings within a moult cycle was observed (Table 1.5).

Evaluating the reproductive performance of test group females fed with the experimental diet, it was observed that out of the total 48 spawns performed by the seven females, three females, numbered 2, 5 and 7, produced 40 spawns. Among the other four females, female 3 and 6 survived only for a short duration and died accidentally while handling. Females 1 and 4 though survived almost till the end of the experiment, showed ovarian development and spawned only after a long period and together gave only five spawns (Table 1.7 and Fig. 1.3 – 1.9). The better response to induced breeding by female 2, 5 and 7 and poor response by female 1 and 4 may be attributed to the genetic variation among individual females. Mc Govern (1988) compared the reproductive performance of individual *P. vannamei* females and observed even when eyestalk ablation was used as stimulus, participation in breeding was extremely variable

among females. Bray *et al.* (1990) found in a population of *P. stylirostris* about 70% of nauplii were produced by only 25% of females. Similar pattern was also reported in populations of other penaeid species. Wyban *et al.* (1990) indicated a great deal of variability among *P. vannamei* females in reproductive performance might be due to genetic factors. Maheswarudu *et al.* (1996) compared the reproductive performance of unablated *P. indicus* females and concluded that females belonging to larger size groups (above 40 g wt) are preferable for induced maturation without eyestalk ablation. In *P. semisulcatus* wild brood stock, individuals measuring between 151-170 mm TL (total length) were found more suitable for seed production than the larger size group (Manickam *et al.*, 1996). Individual size of brood stock was reported as a critical factor for influencing reproductive performance in *P. monodon* (Primavera *et al.*, 1978; and Menesveta *et al.*, 1994), *P. vannamei* (Wyban *et al.*, 1987), *P. stylirostris* (Ramos *et al.*, 1995) and *P. paulensis* (Cavalli *et al.*, 1997). In the present experiment, the poor-performed females (bad breeders) 1 and 4 weighed 25g and 22g respectively, while the better-performed females (good breeders) 2, 5 and 7 weighed 50g, 25g and 40g respectively. Female 5 weighing 25g spawned 13 times whereas female 1 of the same weight spawned only 4 times, indicating size alone is not a factor deciding reproductive performance. At the same time the average spawn size and corresponding number of nauplii were highest for female 2, which was the largest female in the test group (Table 1.7). Thus it can be assumed that size and genetic makeup together decides the response of female shrimp to induced maturation. Thomas (1975) and Bose (1995) observed that in wild *P. semisulcatus* no correlation exists between female prawn size (length and weight) and fecundity.

In the control group, all the females except one were above 35g, while among the test group only three were above 35g. In spite of this difference in size, the test group gave 48 spawns compared to 6 spawns from the control group (Table 1.6and 1.7). Average rate of spawning and average rate of egg production were significantly lesser for control females than for the test females (Table 1.4). The present study clearly demonstrates the influence of a nutritional factor in induction of maturation and obtaining repeated spawning in *P. semisulcatus* for a prolonged period in addition to environmental factors such as salinity, pH, ammonia and nitrite and biological factors like size (age) and genetic variation. Studies by Middleditch *et al.*, (1979, 1980 a and b) showed the need for an increase in polyunsaturated fatty acids (PUFA) in the diet. Bray and Lawrence (1992) roughly outlines the brood stock diet as one containing high percentage of protein (45-65%), with amino acids similar to that of shrimp and lipid portion to meet the demands of essential fatty acids, phospholipids and cholesterol.

The combination of intertidal oligochaetes, squid and clams was reported as a successful maturation diet by Maheswarudu *et al.* (1996) in obtaining repetitive spawning of unablated *P. indicus* and in the ovarian maturation and repeated spawning of ablated *P. monodon* (Dr. G. Maheswarudu personal communication) for prolonged period. In the present study, the intertidal oligochaete *P. bermudensis,* significantly increased the survival of the test group, when fed as dietary supplement, compared to the control group fed squid and clam alone (152.143 ± 11.705 days vs.75.214 ± 4.303 days). The test group produced a total of 54,73,550 eggs and 46,84,800 nauplii in 48 spawnings while the control group produced only 4,12,500 eggs and 3,23,250 nauplii in 6 spawnings. The test animals averaged 6.86

± 2.34 spawns per female, producing an average of 7,81,935 ± 3,12,279 eggs per female, whereas the control females spawned an average of 0.86 ± 0.26 times producing an average of 58,928 ± 18,607 eggs. The average number of nauplii produced per female was also significantly greater in the test group than the control group (669935 ± 271750 vs. 46178 ± 15046). Average number of eggs and nauplii per spawn was also significantly higher for the test group than the control group (92251 ± vs. 68250 ± 3436 and 76391 ± 12381 vs. 37929 ± 10189 respectively). A shortening in the number of days from moulting to spawning was observed for the test females with a subsequent increase in the number of spawns per moult cycle (Table 1.4). A maximum of four spawns per moult cycle were observed for test group, where as the control group spawned only once in a moult cycle (Fig: 1.2). The present experiment clearly demonstrates the success of *P. bermudensis* as a dietary supplement in improving the reproductive potential and survival of captive *P. semisulcatus* brood stock. This can be employed as a advanced method for control reproduction than the commonly used eyestalk ablation technique, which causes mortality of brood stock.

CHAPTER 2

BIOCHEMICAL COMPOSITION AND FATTY ACID PROFILE OF THE INTERTIDAL OLIGOCHAETE *PONTODRILUS BERMUDENSIS*

INTRODUCTION

Since 1970, approximately 23 penaeid species have been matured (and 14 spawned) in captivity. Yet the final goal of captive breeding, - production of consistently high quality nauplii – is not fully realized. Majority of earlier researches on induced maturation in captivity concentrated on eyestalk ablation. But lately researches are focusing on the nutritional requirements of penaeid brood stock without resorting to eyestalk ablation. Many researchers are of the opinion that proper ovarian development, maturation and spawning of penaeid shrimps are related to nutritional status of broodstock diet (Lawrence *et al.*, 1979; Brown *et al.*, 1980; and Middleditch *et al.*, 1980a). Correlation between improved spawning performance and egg and larval quality with brood stock diet have been suggested by Ward *et al.* (1979), Cahu *et al.* (1986 and 1987), Millamena (1989), Bray *et al.* (1990), Lytle *et al.* (1990) and Xu *et al.* (1994b). In spite of this critical importance of proper nutrition in captive breeding, literature related to dietary needs of penaeid broodstock is sparse. Bray and Lawrence (1992), outlined the biochemical characters of diets generally used in shrimp reproduction. The diets have high protein to energy ratios and are high in marine animal source protein (about 45 – 65% protein), often with amino acid profiles similar to shrimp. Another major component of maturation diet is lipids, but precise recommendations for essential fatty acid levels, ratios of n6 (linoleic family) to n3 (linolenic family) fatty acids, phospholipid requirements, and cholesterol

requirements are not available. Brood stock requirements of carbohydrates, minerals and vitamins in diets were not clearly defined (Harrison, 1990). However some works of Chamberlain (1988), Cahu and Fakfakh (1990) and Fakhfakh and Cahu (1990) indicated a high vitamin-E requirement in broodstock diet.

The importance of lipids in maturation diets is quite overwhelming. Lipids, which provide energy as well as essential nutrients, (such as sterols, phospholipids and essential fatty acids) are believed to be one of the key nutritional factors influencing egg hatching rates and larval survival. The rapid rate of ovarian tissue synthesis (mature ovaries may account or 10% or above of female body weight), accelerated rate of ovarian development with eyestalk ablation and limited storage capability of hepatopancreas, the large lipid content of ovarian tissue, and limited ability of shrimp to synthesize polyunsaturated fatty acids predominant in ovaries, inability of shrimp to synthesize sterols and high phospholipid requirement, indicate a higher dietary lipid requirement in adult penaeids for reproduction. Lipids are thought to be the active compounds involved in the reproductive process, given their important role in the physiology of marine animals (Giese, 1966). Compared to the terrestrial animals, marine animals rely more on lipids than carbohydrates for energy reserves (Cowey and Sargent, 1972). Lipids play a major role as nutrient and energy stores and are involved in the formation of biological membranes. They also play a major role in sexual development and breeding, for steroids are used as sex hormones and some essential fatty acids are precursors of prostaglandins (Gurr and James, 1980).

Apart from high level of phospholipids and cholesterol, the major aspect of lipid metabolism to brood stock is the dietary level of fatty acids. Burr and

Burr (1929 and 1930) first demonstrated that a specific unsaturated fatty acid configuration, which could not be synthesized by an animal (the rat), was essential in the diet. Since then much research has been done on the essential fatty acid requirement of both land and aquatic animals. The long carbon chain polyunsaturated fatty acids (PUFA), linoleic (C18: 2n6), linolenic (C18: 3n3), eicosapentaenoic (C20: 5n3) and docosahexaenoic (C22: 6n3) have been shown to be essential for growth in three *Penaeus* species, *P. japonicus, P. merguiensis* and *P. monodon* using the absence of *de novo* synthesis from either ^{14}C-acetate or ^{14}C palmitic acid as a criterion (Kanazawa and Teshima, 1977 and 1981; Kanazawa *et al.*, 1977, 1979 a,b,c,d,e and f; and Deshimaru *et al.*, 1979). In addition to this arachidonic acid (C20: 4n6) was also reported to have only a low degree of endogenous synthesis and has to be originated from the diet (Bottino *et al.*, 1980; Kayama *et al.*, 1980, Lilly and Bottino, 1981; Dall *et al.*, 1991; and Bray and Lawrence, 1992).

Although our knowledge of the nutrient requirements of brood stock shrimp is very limited (Harrison 1990), a number of published observations have shown that the maturation, spawning and egg and larval quality is effected by the types of fatty acids in the diet. Middleditch *et al.* (1979) and Middleditch *et al.* (1980a) compared the fatty acid profiles of ovaries in *P. setiferus, P. stylirostris* and *P. vannamei*, and found that long carbon chain poly unsaturated fatty acids (PUFA), C20: 4n6, C20: 5n3 and C22: 6n3 are predominant in ovaries. They also induced spawning in *P. setiferus* by feeding a supplement of polychaetes rich in these PUFA and suggested, circumstantially, that it was the fatty acid component of bloodworms that was responsible for the ovarian stimulation. Middleditch *et al.* (1980b) surveyed five species of marine annelids, four species of bivalves, two species of crustaceans and a gastropod, and concluded that almost all of these displayed substantially similar fatty acid patterns. Dall *et al.* (1991)

compared the fatty acid profiles of some natural prey species of *P. esculentus* and observed that all of these contained appreciable to high proportions of C20 essential PUFA. The balance of n3 and n6 fatty acids (Lytle *et al.*, 1990) and the possible role of arachidonic acid as a precursor of prostaglandins (Middleditch *et al.*, 1980a and b; and Croz *et al.*, 1988) have been suggested as important factors in shrimp ovarian maturation and spawning. Co-relation between fatty acid levels in ovary and reproductive performance was observed in unablated *P. stylirostris* (Magarelli Jr., 1981). Dietary fatty acid content was found to reflect in spawned eggs (Cahu *et al.*, 1986 and 1987; and Millamena, 1989) and the mean number of eggs per spawn was related to dietary phospolipid content (Cahu *et al.*, 1987).

The importance of non-lipid dietary components for maturation remains largely uninvestigated (Harrison, 1990). Carbohydrate metabolism in maturing penaeids has been poorly studied. Castille and Lawrence (1989) demonstrated that increases in ovarian carbohydrate during maturation occurred in *P. setiferus* and *P. aztecus*. Significant decrease in digestive gland glycogen and simultaneous increase in the glycogen content of ovaries and testes in maturing *Parapenaeopsis hardwickii* have been shown by Kulkarni *et al.* (1979), Kulkarni and Nagabhushanam, (1979) and Nagabhushanam and Kulkarni, (1981). Redon and San-Feliu (1993a) observed an increase in the glycogen content of the ovary in mature females of *P. japonicus*.

Though dietary requirements of carotenoids in shrimp maturation are not determined, it is well known that carotenoids play a specific role in vitellogenesis. The dark pigmentation of the ovary during vitellogenesis is due to the presence of carotenoid pigments. Carotenoids act as a cross link between lipid and protein to stabilize the lipo-protein molecule and

protect eggs from high illumination and solar radiation (Castillo *et al.*, 1982).

Changes in dietary protein requirements of penaeid shrimps during maturation have also not been investigated. Dy-Penaflorida and Millamena (1990) suggested an increase in ovarian protein content from stage I ovary to Stage IV ovary in *P. monodon*, but at the same time observed no significant changes in the amino acid profile. Marangos *et al.* (1989) demonstrated quantitative and qualitative changes in the ovarian and digestive gland free amino acid pools of maturing *P. schmitti*. In *P. vannamei*, Rankin *et al.* (1989) demonstrated active incorporation of amino acids and synthesis of polypeptides in the ovary, at the onset of vitellogenesis. El-Hamid (1989) observed inadequate protein availability following ablation to affect ovarian yolk synthesis in *P. kerathurus*.

Fresh or frozen marine organisms are the commonly preferred broodstock diets than dry feeds. Most researchers use a combination of prepared diet and different fresh feed organisms. Cahu et al. (1986) found that eggs of *P. vannamei* broodstock fed a pelleted diet only were lower in C20: 4n6, C20: 5n3 and C22: 6n3 and higher in C18: 2n6, and had different ratios of n3 to n6 fatty acids compared with diets containing part or all of fresh mussel. Molluscs like mussel, clam, cockle and squid are the most preferable food sources for penaeid brood stock. Other feed items commonly used are fresh or frozen marine worms, mysids, shrimp, fish, brine shrimp, shark, troca and krill. Diets containing multiple fresh suppliments were generally reported to out perform single component supplements. Chamberlain and Lawrence (1981a) found that growth and maturation of *P. vannamei* and *P. stylirostris* were enhanced with a combination diet consisting of squid, shrimp, blood worms and clams over any single food diet. But an all-fresh diet without any prepared feed

supplement was always found to elicit the highest maturation response in penaeids (Galgani *et al.*, 1989a; Bray *et al.*, 1990 and Luis and Ponte, 1993).

One of the main criterion deciding the selection of a particular fresh feed item as maturation diet is its regional availability. In the Western Hemisphere, two species of polychaete worms, the Maine bloodworm (*Glycera dibranchiata*) and the Panama bloodworm (*Americonuphis reesei*), are commercially available and form about 25 % to 35% (dry weight basis) of the maturation diet of *P. vannamei* (Bray and Lawrence, 1992). In the South Pacific AQUACOP (1977a) observed that fresh *Trocus niloticus* has a positive effect on the maturation and egg viability of several species of penaeids.

Marine annelids are an established group of fresh feed item in shrimp maturation diet. Addition of *G. dibranchiata* to other fresh feed components or pellets was found to improve reproductive performance of *P. vannamei* (Gomez and Arellano, 1987; and Ogle, 1988) and of *P. setiferus* (Middleditch et al., 1979 and 1980b; Chamberlain and Lawrence, 1981a; and Brown *et al.*, 1979). Croz *et al.* (1988) obtained successful maturation in captive *P. vannamei* and *P. stylirostris* by using the Panama bloodworm *A. reesei.* The rag worm (*Neries diversicolor*) was found to induce gonadal maturation and spawning in *P. kerathurus*, when fed either as single or as composite feed (Luis and Ponte, 1993). Lytle *et al.* (1990) comparing the fatty acid profiles of the sand worm, *N. viridens* with *G. dibranchiata,* found similar levels of total PUFA and therefore concluded that sandworms should be a successful maturation diet for *P. vannamei.* Marchiori and Boff (1983) used annelid worms along with clam and fish for the induced maturation of eyestalk ablated *P. paulensis.* All

the marine annelids listed so far as successful maturation diet for penaeid shrimps belong to Polychaetes.

Several species of oligochaetes are reported as food for freshwater and marine invertebrates and fishes and also as fishing bait. Tidwell *et al.* (1997) observed that the naturally occurring oligochaetes in aquaculture ponds closely match the biochemical composition of juvenile *Machrobrachium rosenbergii*. Tubificids are important to freshwater aquaculture particularly because of their high food value, 5575 cal/g dry weight (Cummins and Waycheck, 1971 in Giere and Pfannkuche, 1982), which makes them nutritious feed for fish (Marian, 1982). The lugworms (*Arenicola* species) are famous as fish bait in U.S.A., and as food for aquarium fishes and cultured sturgeons. Species of white worm *Enchytraeus* and the common earthworm, *Lumbricus* are preferred food items for larger cultured invertebrates and fishes. Swingle (1961) details the commercial production of fish worm (red worm), which are used as fish bait and food for cultured animals.

Earthworms are renowned as the best natural fishing baits. Sabine (1983) suggested the consumption of earthworms as food by domestic animals and even by human beings. Earthworms form the natural feed to many amphibians like *Bufo bufo* (Lescure, 1966), *Rana temporaria* (Smith, 1951), *Salamandra salamandra* (Lopez *et al.*, 1979) and *Thamnophis butleri* (Catling and Freedman, 1980). Feeding experiments with earthworm meal to chicken (Harwood, 1976; Yoshida and Hoshii, 1978; McKada *et al.*, 1979; and Taboga, 1980), growing pigs (Harwood and Sabine, 1978) and to mice and rats (Mc Inroy, 1971; and Schulz and Graff, 1977) indicated their importance as a better source of protein compared to conventional protein meals. Stafford and Tacon (1984) in their growth trial experiments with the rainbow trout, *Salmo gairdneri*

demonstrated significant improvement in fish fed exclusively on blanched earthworms of the species *Lumbricus terrestris* and *Allolobophora longa*. They also found that dried earthworm meal derived from *Eisenia foetida* could adequately replace the fishmeal component of a formulated trout pellet at 5-30% by weight inclusion without adversely affecting fish growth. They reported that the chemical and nutritional characteristics of various species of earthworms were similar to fishmeal and the amino acid and polyunsaturated fatty acid profiles of earthworms were particularly suitable for use in commercial fish production. Other dietary trials with earthworms in fishes include those by Tacon (1981) in young eels; Tacon *et al.* (1983) and Hilton (1983) in Trout; and Guerrero (1983) in Tilapia. The only observation of intertidal oligochaete as a successful shrimp brood stock dietary supplement was by Maheswarudu *et al.* (1996) in the repeated spawning of unablated *P. indicus*. The intertidal oligochaete, *P. bermudensis* as a dietary supplement was observed to improve the reproductive performance of unablated *P. semisulcatus*, during the maturation experiments conducted under the present study.

Gani (1985) compared the biochemical constituents of earthworm meal made from *Lampito mauritii* with four commercially available fish feeds and found that the earthworm meal was superior to other feeds. Biochemical analysis of the earthworm *L. mauritii* by various investigators revealed that glycogen content was between 2% and 7.5% (Saroja and Rao, 1965), protein content was between 24.5% and 73.76% and lipid content was around 10% (Dash *et al.*, 1977). According to Lawrence and Willar (1945) the protein content of various species of earthworms varied between 53.5% and 71.5% (dry weight basis). Bolton and Philipson (1976) found that the calorific value of earthworms was equal to that of mammalian muscle. Taboga (1980) reported the high protein content of

earthworms and found them rich in essential amino acids, including those containing sulphur. The amino acid composition of earthworms was found to be similar to that of mammalian muscle and its metabolic composition was found to be suitable for the growth of other animals and even human beings as mammalian meat (Sabine, 1978 and 1983). Earthworm tissues also contain specific nutritive compounds like triglycerides (Hansen and Czochanska, 1974) and free fatty acids (Naya and Kotake, 1967). Clive and Niederer (1988) reported a preponderance of long chain essential fatty acids and an excellent range of vitamins and minerals in earthworm tissues.

Variations in organic constituent levels were observed during the different growth stages of *L. mauritii* (Patra and Dash, 1973; Dash *et al.*, 1977; and Alawdeen and Ismail, 1986). Since the population of earthworm species at anytime has been reported to be made up of young immature, well grown immature (adolescent), mature and senescent individuals (Edwards and Lofty, 1972), it is desirable to know the nutritional value of different stages of growth so that the nutritionally rich stage could be harvested while other stages are allowed to maintain and build up the population (Alawdeen and Ismail, 1986).

The present chapter attempts to characterize the biochemical composition of the three stages - juveniles, non-clitellates and clitellates - in the lifecycle of the intertidal oligochaete *P. bermudensis*. The fatty acid profile of a composite sample representing the three growth stages was also analyzed through Gas chromatography.

MATERIALS AND METHODS

A. Biochemical analysis:

Sample collection:

Live *Pontodrilus bermudensis* were collected from the intertidal areas along the coastline of Gulf of Mannar, Mandapam and hand sorted into juveniles (<3.5 cm), non-clitellates and clitellates (both >3.5 cm). The worms were then defecated on moist filter paper inside glass troughs for 24 hrs and were then blotted to remove excess moisture.

Estimation of moisture content:

The wet weights of the tissues were taken accurately and the samples were gradually dehydrated to a constant weight in the hot air oven at 60° C. The moisture content was calculated gravimetrically as the difference in the wet weight and the dry weight of the tissue and was expressed as percentage of wet weight.

$$\% \text{ Moisture content} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight of the tissue}} \times 100$$

Estimation of total protein:

The total protein was estimated by the method of Lowry *et al.* (1951) using Bovine Serum Albumin as standard. About 400mg of dry tissue was accurately weighed and homogenised with 20 ml distilled water. To 1ml of

aliquot of the homogenate 1ml of 5% TCA was added (deprotenising agent) and centrifuged at 5000rpm for 5 minutes. The precipitate obtained was digested in 0.5 ml 0.1 N NaOH and taken for estimation. To a 0.5 ml aliquot from this 5 ml alkaline copper reagent was added and mixed well. After 10 minutes, 0.5 ml Folin-phenol reagent was added and mixed rapidly. Optical density (OD) was read at 660 nm in an ECIL UV spectrophotometer along with reagent blanks. A standard graph was plotted with different dilutions of the working standard in the X-axis and the corresponding Optical Densities (OD) in the Y-axis. Concentration of protein in the sample was calculated in mg% by comparing the OD obtained for the sample with the values in the standard graph.

Estimation of total lipids:

Total lipid content of the worms was estimated gravimetrically by the method of Folch *et al.* (1957). About 500 mg dry sample was accurately weighed and homogenized for 1 minute with 10 ml methanol, then 20 ml of chloroform was added and the process continued for a further 2 minutes. The mixture was filtered through a Buchner funnel and the solid residue resuspended in chloroform-methanol (2:1 v/v, 30 ml) and homogenized for 3 minutes. After filtering, the solid residue was washed once again with chloroform (20 ml) and methanol (10 ml). The combined filtrates were measured and one quarter of the total volume of the filtrate of 0.88% potassium chloride in water was added to facilitate phase separation. The whole extract was transferred to a separating funnel, shaken thoroughly and allowed to separate overnight at low temperature. The lower layer was collected and one quarter of its volume of water: methanol (1:1) was added and the washing repeated. The bottom layer containing the purified lipid was filtered through anhydrous sodium

sulphate and was evaporated to dryness in a water bath. The weight of the lipid obtained was determined gravimetrically using a Metlar monopan balance.

Estimation of total carbohydrates:

Total carbohydrates – simple sugars, oligosaccharides and polysaccharides were estimated using the Phenol - Sulphuric acid method (Dubois *et al.*, 1956). About 400 mg of dry sample was accurately weighed and deproteinized using 80% ethanol. To 1ml aliquot of the supernatant 1ml of 5% phenol was added. Then 5 ml of concentrated H_2SO_4 was added directly against the liquid surfaces to obtain good mixing. After a stable orange-yellow colour was developed the solution made up to 11 ml with distilled water and allowed to cool to room temperature. Optical density was measured at 490 nm in an ECIL UV spectrophotometer along with reagent blanks. D-Glucose was used as standard and standard graph was plotted with different dilutions of the working standard in the X-axis and the corresponding ODs in the Y-axis. Concentration of carbohydrate in the sample was calculated in mg% by comparing the OD obtained for the sample with the values in the standard graph.

Estimation of total carotenoids:

Total carotenoids were estimated following the method of Olson (1979). About 1 gm of fresh tissue was weighed accurately on an aluminum foil and placed in clean dry screw capped 10ml vial containing 2.5gm of anhydrous sodium sulphate. Tissue was gently mashed using a glass rod and 5ml of chloroform was added to the vial to extract carotenoids. The

vial was sealed and kept overnight at 0°C in the dark. A blank was also run with 5 ml of chloroform. For total carotenoid estimation 0.3 ml aliquots of chloroform extracts were drawn from the vial and diluted to 3 ml with absolute ethanol. Blank was treated in the similar manner. Optical density was read at 290, 350, 380, 450, 475 and 500 nm. The readings were plotted on a graph. Wavelength with maximum absorption (450 nm) was used for calculation.

$$\text{Total carotenoids } (\mu g/gm) = \frac{\text{absorption at 450nm} \times \text{dilution factor}}{0.25 \times \text{sample weight in gm}}$$

Dilution factor = 50

Extinction coefficient = 0.25

Estimation of ash content:

A preweighed sample of oven dried powdered tissue was ignited in a silica crucible for 5 hours at 600°C in a muffle furnace till all the organic matter was burnt out leaving no carbon residue. The ignited content was weighed and the difference in weight gave the ash content of the tissue. The percent ash content of the tissue was calculated as follows

$$\text{Ash mg\%} = \frac{\text{ash weight}}{\text{dry weight of the tissue}} \times 100$$

B. Analysis of Fatty acid profile by Gas Chromatography:

Sample collection and transportation:

Samples of live *Pontodrilus bermudensis* were obtained from the intertidal regions along the coastline of Gulf of Mannar, Mandapam. The worms were transported live in earthern pots filled with sand and covered with wet gunny bags, from Mandapam Regional Centre of Central Marine Fisheries Research Institute (CMFRI) to Central Institute of Fisheries Technology (CIFT), Kochi. The extraction of total lipids, saponification, esterification and analysis of fatty acids profiles in Gas Chromatogram (GC) were done at the Biochemistry and Nutrition Division of CIFT, Kochi. Only fresh samples were used for analysis.

Sample protection:

Several precautions were taken to ensure that no degradation of the lipids occurred during storage, extraction and saponification. All solvents were flushed with N_2 immediately before use to remove dissolved O_2 to prevent oxidative degradation. Likewise, samples requiring storage were flushed with N_2 before being placed in freezers. In addition, an antioxidant 2,6-di-tert-butyl-p cresol (BHT) was added in a concentration of 0.005% (w/v) to the extracting solvents to prevent oxidative degradation of unsaturated lipids.

Sample preparation:

Fresh, live worms were used for analysis. Total lipids were extracted from the tissues by the method of Bligh and Dyer (1959). After saponification, saponifiable materials were recovered and fatty acids were converted to fatty acid methyl esters (FAME).

Lipid extraction:

The worms were defecated on moist filter paper inside glass troughs for 24 hours and were then blotted to remove excess moisture. About 50 gm of worms were accurately weighed and homogenized thoroughly. Total lipid was extracted by blending the homogenized tissue in a blender with a solvent mixture consisting of 15 volumes of chloroform-methanol (2:1v/v). The mixture was filtered through a Buchner funnel, transferred to a measuring cylinder and noted the total volume. To this about 0.2 of its volume (v/v) of water was added to facilitate phase separation and the whole extract was transferred to a separating funnel, shaken thoroughly and allowed to separate overnight at low temperature in nitrogen atmosphere. From the resultant biphasic solution, bottom chloroform layer containing the purified lipid was collected and dried with anhydrous sodium sulphate till the solution was clear and filtered. Total volume of the chloroform extract was noted and a small quantity was taken in a pre-weighed vial and the lipid content was estimated by gravimetry. The chloroform extract was evaporated in a vacuum flash evaporator and lipid was stored in a small volume of chloroform in a deep freezer till further analysis.

Saponification:

About 0.5g of lipid sample was accurately weighed and evaporated to dryness in a round bottom flask. To this 30 ml methanol and 1.5 ml 150% (15 g in 100 ml) KOH were added and refluxed for 2 hours (Lipid hydrolyzed in alcoholic KOH). The contents of the flask were cooled and extracted with 30 ml Petroleum Ether (60°-80°), in a separating funnel to remove the non-saponifiable matter (NSM). On separation, the bottom aqueous layer contained fat and top Petroleum Ether (PE) layer contained NSM. Both the layers were collected separately and the whole procedure was repeated thrice.

The combined aqueous layer was acidified with concentrated HCl (about 1-2ml HCl was added drop by drop till the solution becomes acidic and tested with pH paper for acid) and extracted again with 30 ml PE to recover the fatty acids. The ether extract was washed with water, dried over anhydrous sodium sulphate and filtered.

Esterification:

The fatty acid portion was taken in a round bottom flask, solvent was evaporated and to this 15 ml BF_3 MeOH was added. The contents of the flask were refluxed for 6 minutes, cooled and 6 ml of saturated sodium chloride solution was added. From this the methyl esters were separated by extraction (4 times) with 25 ml PE. The combined ether extract was washed with water, dried over anhydrous sodium sulphate, filtered and was evaporated in a rotary evaporator. The residual Fatty Acid Methyl Esters (FAME) were redissolved in Chloroform (2–3 ml), collected in

small Teflon capped vials, flushed with N_2 and stored in deep freezer until further analysis.

Gas chromatograph analysis:

Fatty acid methyl esters were separated and characterized by capillary gas chromatography (GC) using a Chrom Pak 9001 equipped with flame ionization detector and fitted with a 6' x 1/8" i.d. stainless steel column packed with Chromosorb H.P. (Mesh size 80-100) and liquid phase OV 275 (10%). Nitrogen was used as carrier gas (13.5ml/min flow rate). Using a hypodermic syringe, 0.5–1 µl of the sample was injected through the injection port. The temperature programme during the sample run was as follows – injection temperature 230°C and detection temperature 240°C. Column initial temperature was 100°C, which was programmed to increase at the rate of 3°C/min till 160°C and then 5°C/min for the rest. Total runtime was 50 minutes. Detector used was Flame Ionization Detector (FID) with H_2 at the rate of 50 ml/min. All data were processed using the mosaic software supplied by Chrom Pak with arithmetic means of two injections computed for each sample. Fatty acid methyl ester identification was obtained by comparison of retention times (relative to a reference standard) with those of authentic standards (FAME standards from SIGMA or Aldrich).

Statistical data treatment:

Analysis of variance was used to find whether there are any significant differences between the biochemical constitutions of the three growth stages of *P. bermudensis*. For the fatty acid profile mean and percent relative standard deviation (% RSD) values were calculated.

RESULTS

A. Biochemical analysis:

Table 2.1 summarizes the results of biochemical analysis of the littoral oligochaete *Pontodrilus bermudensis*. The three growth stages – juveniles, non-clitellates and clitellates – exhibited significant differences in the values of various components. Non-clitellates registered the highest percentage of moisture, compared to those of the other stages. The percent of moisture was significantly lower for the juveniles than the adults. Carbohydrates were found to be concentrated in clitellates and juveniles. Carbohydrates being energy source is quite necessary for growth and reproduction.

Protein content was above 45% (dry weight basis) in all the stages. Non-clitellates were found to posses a considerably higher concentration of proteins, though there was no significant difference between the protein content of the three stages. Fat seemed to be accumulated in juveniles. Among the adults, the clitellates were found to possess a higher concentration of lipids, obviously as a source of energy and also as a source of essential nutrients such as sterols, phospholipids and fatty acids, for reproduction. An inverse relationship was evident between the water content and total lipid content of the animals at all stages. Significantly higher concentration of total carotenoids was observed in the clitellates while compared to that of the non-clitellates. Juveniles exhibited a higher ash content than the adult stages.

Table 2.1: Biochemical composition of the three growth stages of *P. bermudensis*

Stage	Moisture content (% body wt.)	% mg dry weight			Total carotenoids (µg/gm wet weight)	Ash
		Carbohydrate	Protein	Lipid		
Juveniles	68.53±1.44	0.9724±0.082	46.23±2.19	15.33±0.65	Not analyzed	6.22±0.37
Non-clitellates	78.49±1.25	0.8260±0.052	51.08±1.63	11.96±0.73	4.06± 0.45	5.12±0.30
Clitellates	76.08±1.68	1.0946±0.073	49.54±1.55	12.08±1.02	6.33 ±0.44	5.57±0.22

Table 2.2: Analysis of variance of moisture content

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
juvenile	15	1027.97	68.53133	31.12257
nonclitellate	15	1177.43	78.49533	23.54333
clitellate	15	1141.23	76.082	42.66087

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	810.5902	2	405.2951	12.49281	5.53E-05	3.219938
Within Groups	1362.575	42	32.44226			
Total	2173.165	44				

SE	2.079816
t value	1.96
CD	4.076439
F1-F2	9.964s
F2-F3	2.413333ns

Moisture content was significantly less in juveniles compared to those of other two stages.

Table 2.3: Analysis of Variance of total carbohydrates

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*	
juvenile	15	14.5864	0.972427	0.1003277	0.316745
nonclitelate	15	12.3905	0.826033	0.0405605	
clitellate	15	16.419	1.0946	0.0816046	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.542427	2	0.271213	3.6569293	0.034347	3.219938
Within Groups	3.114899	42	0.074164			
Total	3.657326	44				

SE	0.099441
t value	1.96
CD	0.194905
F1-F2	0.146393ns
F2-F3	0.268567s

Significantly higher concentration of carbohydrates was observed in clitellates compared to that of non-clitellates.

Table 2.4: Analysis of Variance of total proteins

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Juvenile	15	693.43	46.22867	72.18251
Non-clitellate	15	766.24	51.08267	39.77879
Clitellate	15	743.12	49.54133	36.02464

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	184.5539	2	92.27696	1.870657	0.16663	3.219938
Within Groups	2071.803	42	49.32865			
Total	2256.357	44				

No significant difference was found between the three stages in total protein content.

Table 2.5: Analysis of Variance of total lipids

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
juvenile	16	245.23	15.32688	6.77193
nonclitellate	16	191.38	11.96125	8.435665
clitellate	16	193.23	12.07688	16.71334

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	116.8176	2	58.4088	5.489388	0.007358	3.20432
Within Groups	478.8141	45	10.64031			
Total	595.6317	47				

SE	1.153273	
t alpha	1.96	
CD	2.260415	
F1-F2	3.365625	significant
F2-F3	0.115625	n.significant

Juveniles possess significantly higher concentration of total lipids than the non-clitellates and clitellates.

Table 2.6: Students t- Test comparing the total carotenoids

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	4.055	6.3325
Variance	2.039914	2.006336
Observations	8	8
Pooled Variance	2.023125	
Hypothesized Mean Difference	0	
df	14	
t Stat	-3.20241	
P(T<=t) one-tail	0.003195	
t Critical one-tail	1.761309	
P(T<=t) two-tail	0.00639	
t Critical two-tail	2.144789	

Significantly higher concentration of total carotenoids was found in clitellates compared to that of non-clitellates

Table 2.7:Analysis of Variance of ash content

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	15	93.33	6.222	2.095503
Column 2	15	76.79	5.119333	1.383235
Column 3	15	83.53	5.568667	0.744912

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.223093	2	4.611547	3.275517	0.04765	3.219938
Within Groups	59.13111	42	1.407883			
Total	68.3542	44				

No significant difference was observed between the three stages in the ash content

B. Fatty acid analysis:

The fatty acid profile of *P. bermudensis* is presented in Table 2.8. The predominant fatty acids identified in the oligochaete worm were C14: 0, C18: 1n9 and C20: 4n6. These three were present in concentrations above 10%. C16:0, C18:0, C20: 1n9, and C18: 2n6 fatty acids were also present in considerably high quantities. The total saturated fatty acids amounted to 35.18%, total monounsaturated fatty acids to 24.62% and total polyunsaturated fatty acids (PUFA) to 24.05%, of the total fatty acids. Due to lack of corresponding standard fatty acid methyl esters, only 83.86% of the total fatty acids could be identified. The unidentified fatty acids amounted to 16.14%.

Among the saturated fatty acids, C14: 0 was the major component, followed by C16:0 and then C18:0. Longer chain saturates were either absent or were unidentified. The most abundant monounsaturated fatty acid was C18: 1n9. C20: 1n9 and C16:n7 were also present in considerably high quantities. Longer carbon chain C24: 1 was present around 2%.

C20: 4n6 (arachidonic acid) was the major PUFA detected in *P. bermudensis*. Unlike marine invertebrates, a preponderance of n6 PUFA compared to n3 PUFA was observed. C18: 2n6 (linoleic acid) was also present in fairly good quantities. In the n3 series, four fatty acids were identified. Compared to marine organisms fairly good concentrations of C18: 3n3 (0.62%) and C18: 4n3 (1.58%) and low concentrations of C20: 5n3 (2.48%) and C22: 6n3 (1.19%) were detected. Total unsaturated fatty acids (monounsaturated and polyunsaturated) amounted to 48.68%. Ratio of unsaturated to saturated fatty acids was1.38: 1 and n3 to n6 was 0.32: 1.

Table 2.8 : Fatty acid profile of *Pontodrilus bermudensis.*

Fatty acids [a]	Relative % composition [b]		Absolute concentration [b]	
	%	% RSD [c]	µg/g	% RSD [c]
Saturates				
C14:0	12.93	0.12	431.83	6.15
C15:0	4.22	0.11	141.03	6.97
C16:0	9.60	0.14	322.22	14.74
C17:0	2.29	0.14	76.83	12.21
C18:0	6.14	0.05	206.89	11.40
Total saturates	35.18	0.11	1178.80	9.91
Monounsaturates				
C16: 1n7	4.27	0.06	143.55	8.04
C18: 1n9	11.48	0.08	386.52	12.64
C20: 1n9	6.68	0.05	224.61	9.50
C24: 1	2.20	0.18	73.48	16.93
Total Monounsaturates	24.63	0.08	828.16	11.33
Polyunsaturates				
C18: 2n6	8.19	0.07	275.92	12.25
C18: 3n3	0.62	0.30	20.50	23.82
C18: 4n3	1.58	0.12	52.69	8.25
C20: 4n6	10.01	0.06	337.06	12.02
C20: 5n3	2.48	0.05	83.22	6.64
C22: 6n3	1.19	0.12	39.70	9.05
Total polyunsaturates	24.06	0.08	809.09	11.45

Identified	83.86	0.09	2816.06	6.14
Unidentified	16.14	0.17	544.20	21.20
Σ Fatty acids	—	—	3360.25	6.69
Σ n3	5.86	0.11	196.11	9.35
Σ n6	18.20	0.06	612.98	12.13
Unsaturates	48.68	0.08	1637.25	11.39
Σ unsat./ sat.[d]	1.38	0.67	1.39	114.88
Σ n3/n6	0.32	1.72	0.32	77.12

[a] Fatty acid symbols : Cx: y, x = no. carbons. y = no. double bonds.

[b] % of total fatty acid fraction; μg/g wet tissue weight.

[c] % RSD = standard deviation/ mean.

[d] (total polyunsaturates + total monounsaturates) / total saturates.

DISCUSSION

Dash *et al.* (1977) reported earthworms as excellent feed for fish and poultry. Biochemical analysis of the earthworm *L. mauritii* by various investigators has revealed that the glycogen content varies between 2% and 7.5% (Saroja and Rao, 1965); protein content between 24.5% and 73.76%; lipid content is at 10% (Dash *et al.*, 1977); and the free amino acid level in the coelomic fluid varies between 7.5 ±3.0 and 13.8 ±3.7 mM glycine equivalent (Rao, 1963). Stolte (1924), Eckert (1934) and Avel (1959) found that rate of growth is closely proportional to organic reserve level in the feed of oligochaetes. Therefore it may be expected that the different stages of growth of oligochaetes possess different concentrations of biochemical constituents. Variations in the levels of biochemical constituents during immature and mature stages of *L. mauritii* and *P. excavatus* were reported (Patra and Dash, 1973; Dash *et al.*, 1977; Alawdeen and Ismail, 1986; and Ismail, 1997). The results of the present investigation also reveal such a variation in the biochemical components during the growth of *P. bermudensis*.

Adequate diet is quite essential in the successful maturation and spawning of penaeid brood stock. In spite of this, relatively very few controlled experiments have been carried out on the nutritional requirements of female brood stock (Corbin *et al.*, 1983 and Primavera, 1985). Comparative literature concerning dietary requirements especially for penaeid reproduction is almost non-existent (Bray *et al.*, 1990 and Harrison, 1990). In the present study, the experimental diet (*P. bermudensis*, squid and clam) was found to influence the reproductive performance of *P. semisulcatus* significantly than the standard diet (squid

and clam). The carbohydrate level of *P. bermudensis* was observed to range from 0.84% to 1.07% during the three stages of growth. Saroja and Rao (1965) reported a glycogen content of 2-7.5% in the earthworm *L. mauritii*. Specific requirements of dietary carbohydrates in penaeid maturation are yet to be clearly defined. Castille and Lawrence (1989) observed an increase in ovarian carbohydrate content during maturation in *P. setiferus* and *P. aztecus*. One of the major components of crustacean yolk is vitellin, which is a lipoglycoprotein. Kulkarni *et al.* (1979), Kulkarni and Nagabhushanam (1979) and Nagabhushanam and Kulkarni (1981) observed a significant increase in glycogen content in the maturing gonads of *P. hardwickii*. Mohammed (1989) recorded 2.8% (dry weight) carbohydrate in the yolk of *P. indicus*. In *P. semisulcatus*, ovarian carbohydrate was shown to increase from 0.01% in stage I to 0.63% in stage IV (Bose, 1995). In the present study the recorded total carbohydrate level in *P. bermudensis* was low compared to that of *L. maurti* reported by Saroja and Rao (1965). But it was source for carbohydrate along with squid and clam for both test and control groups.

The non-clitellate and clitellate stages of *P. bermudensis* recorded a total carotenoid content of 4.06± 0.45 and 6.33± 0.44 (mg% wet weight) respectively (Table 2.1). Carotenoids are thought to play an important role in shrimp reproduction. Mohamed and Diwan (1992) observed a steady build up of carotenoids in the ovary of *P. indicus* during maturation and inferred that it may be used for the formation of the glyco-lipo-caroteno-protein. An increase in the total carotenoid content of ovary was reported in *Plesiopenaeus edwardsianus* (Establier, 1966) and in *P. semisulcatus* (Bose, 1995). Astaxanthin is considered to be the major carotenoid of Crustacea, comprising about 90% of the total pigments in *P. japonicus* (Ishikawa *et al.*, 1966). The dark green colouration of ripe ovary in *P.*

semisulcatus is apparently due to astaxanthin pigment as green in blue complexes are often found to contain astaxantin as the prosthetic group (Tanaka *et al.*, 1976 and Castillo *et al.*, 1982). It may be attributed that *P. bermudensis* contributed required carotenoids either directly or indirectly for test group *P. semisulcatus* to perform repetitive spawning for prolonged period.

Above 45% protein (mg% dry weight) was recorded for all the three stages of *P. bermudensis* in the present study (Table 2.1). Earthworm tissue was reported to be high in protein and rich in essential amino acids (Sabine, 1983), including those containing sulphur (Taboga, 1980). Earthworm meal was suggested as a better source of protein than conventional protein meals by many researchers (Mc Inroy, 1971; Harwood, 1976; Schulz and Graff, 1977; Harwood and Sabine, 1978; Yoshida and Hoshii, 1978; Mc Kada *et al.*, 1979; and Taboga, 1980). The amino acid composition of earthworm protein as reported by Taboga (1980) is given in Table 2.9.

Table 2.9: Amino acid composition of earthworm protein (g/100g of protein) (Source: Taboga, 1980)

Amino acid	Concentration (g /100g protein)
Alanine	5.4
Arginine*	7.3
Aspartic acid	10.5
Cysteine	1.8
Glutamic acid	13.2
Glycine	4.3
Histidine*	3.8
Iso leucine*	5.3
Leucine*	6.2
Lysine*	7.3
Methionine*	2.0
Phenylalanine	5.1
Proline	5.3
Serine	5.8
Threonine*	6.0
Tryptophan*	2.1
Tyrosine	4.6
Valine*	4.4

* **Essential amino acids**

About 80% of crustacean vitellin was thought to be protein. In penaeids, the ovary plays a major role in yolk protein synthesis with some synthesis and storage of precursors in the hepatopancreas (Yano and Chinzei, 1987; Quackenbush, 1989 a and b; and Browdy *et al.*, 1990). Rankin *et al.* (1989) using autoradiography observed active incorporation of amino acids at the onset of vitellogenesis and synthesis of many polypeptides in the ovary of *P. vannamei*. Protein was found to be the most abundant constituent of *P. japonicus* ovary (Redon and San-Feliu, 1993a). Mohammed (1989) observed 39.27% (dry weight) protein in the mature yolk of *P. indicus*. An increase in ovarian protein content from stage I to stage IV and a decrease in stage V was observed in *P. monodon* (Dy-Penaflorida and Millamena, 1990) and in *P. semisulcatus* (Bose, 1995). Ovary of P. *semisulcatus* was found to contain about 54.22% of protein at stage IV. Not much attention has been directed to optimize the dietary protein requirements in penaeids during maturation. In ablated *P. kerathurus* inadequate dietary protein levels were shown to affect ovarian yolk synthesis (El-Hamid, 1989). Bray and Lawrence (1992) suggested penaeid maturation diet to be high in marine animal source protein (45-65%), with amino acid profile similar to shrimp and have high protein: energy ratios. Dall *et al.*, (1991) observed very high protein content (52-62%) in some prey species of *P. esculentus*. In the present study *P. bermudensis* along with the other two feed supplements, squid and clam, clearly provided the protein requirement of the brood stock. Squid was reported to contain high level of protein (approximately 60%), with amino acid profiles similar to that of shrimp protein (Gomez and Arellano, 1987; Galgani *et al.*, 1989 a and b; Bray *et al.*, 1990 and 1992; and Menesveta *et al.*, 1994). Clam meat was reported to contain protein fractions helping in maturation (Kanazawa, 1990).

In the present study lipid content of the three stages of *P. bermudensis* was found to range between 11.96-15.51% (dry weight) (Table 2.1). Earthworm tissues were reported to contain fairly good amounts of triglycerides (Hansen and Czochanska, 1974) and free fatty acids (Naya and Kotake, 1967). Most of the controlled experiments regarding the nutritional aspects of captive maturation in penaeids have concentrated on lipids. The need for attention to the lipid portion of the breeders diet is clear. Lipids act as a source of energy, essential fatty acids and cell constituents in the process of spawning, embryo genesis, hatching and early development of larvae in aquatic animals (Holland, 1978 and Takashima, 1974).

Mature ovaries (which may account for about 10% of total female weight) were observed to contain large amount of lipids. Lipids constituted approximately 17.5% (dry weight) of the ovary in wild collected *P. setiferus* and in captive females it was 20.1% (dry weight) (Chamberlain and Lawrence, 1981b; Castille and Lawrence, 1989; and Middleditch et al., 1980a); in *P. aztecus* it was 19.1% and approximately 17.5% of ovary dry weight respectively (Chamberlain and Lawrence, 1981b; Castille and Lawrence, 1989); in *P. indicus*, 18.7-21.4% and 29.14% dry weight respectively (Read, 1977; Mohammed, 1989); and in *P. japonicus* 7.3% and 10.1% wet weight respectively (Guary *et al.*,1974; Teshima and Kanazawa, 1983). Bose (1995) observed a sharp increase in ovarian lipid from 6.66% at stage I to 22.93% at stage IV in *P. semisulcatus*. The limited storage capacity of the digestive gland also suggests a strong requirement of lipid from immediate ingestion (Chamberlain and Lawrence, 1981b; Castille and Lawrence, 1989). By changing the lipid content of a dry formulated diet that was fed in combination with a fresh-frozen component, Bray *et al.* (1990), suggested a dietary lipid level of

10.3% -11.1% (dry weight basis) as appropriate for the maturation and spawning of ablated *P. stylirostris*. In the present study it is very clear that *P. bermudensis* being rich in lipid content (11.0 – 16.0%), contributed lipid in adequate level to perform repetitive spawning by the test group females for prolonged period.

One of the major aspects of lipid metabolism in maturation of penaeid shrimp is the dietary level of fatty acids. The long carbon chain polyunsaturated fatty acids (PUFA) – linoleic (C18: 2n6), linolenic (C18: 3n3), arachidonic (C20: 4n6), eicosapentaenoic (C20: 5n3), and docosahexaenoic (C22: 6n3) – were suggested to be involved in the shrimp reproductive process (Kanazawa and Teshima, 1977 and 1981; Kanazawa *et al.*, 1977; 1979 a,b, c, d, e and f; Deshimaru *et al.*, 1979; Middleditch *et al.*, 1979 and 1980 a and b; and Lawrence *et al.*, 1979). Among these, the n3 group of fatty acids, particularly eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids are considered to have the highest nutritive value of essential fatty acids (Kanazawa *et al.*, 1978 and 1979). Several studies which documents the fatty acid composition of the organs of different shrimp species have demonstrated that, throughout ovarian maturation, ovarian lipids contained higher proportions of C20: 5n3 and C22: 6n3 than that of hepatopancreas (Teshima and Kanazawa, 1983; Jeckal *et al.*, 1989; and Ji and Xu, 1992). Xu *et al.* (1994a) indicated that for *P. chinensis* juveniles, the order of nutritional value of purified fatty acids was docosahexaenoic acid, followed by arachidonic acid, linolenic acid and then linoleic acid. Mourente *et al.* (1990) showed that 65% of the fatty acids of the total ovarian lipids are incorporated into egg and embryos during ovarian maturation of *P. kerathurus*. The reproductive performance and offspring quality of several species of penaeids were found linked to dietary n3 PUFA levels. Middleditch *et al.* (1980a) induced

spawning in *P. setiferus* while feeding a supplement of polychaetes and suggested that it was the PUFA component of blood worms that was responsible for the ovarian stimulation. Magarelli Jr. (1981) observed that the level of C22: 6n3 was possibly correlated with hatch in the F1 but not P1 population in unablated *P. stylirostris*. Improved reproductive performance, egg quality and hatching efficiency of *P. monodon* was shown to be correlated with higher n3 (28.6%) content and higher n3/n6 fatty acid ratio (1.81) in the brood stock diet (Millamena, 1989 and Millamena *et al.*, 1986). Cahu *et al.* (1986 and 1987) pointed out a correlation between the fatty acid composition of ripe ovary and spawned eggs with the fatty acid composition of brood stock diet in penaeids. Xu *et al.* (1994b) showed the correlation between the content of C20: 5n3 in the egg and egg production of the brood stock; and between the levels of C22: 6n3 in the egg and the hatching rate in *P. chinensis*.

In the review of reproduction of *Penaeus* species in captivity, Bray and Lawrence (1992) suggested that the fatty acid profile of shrimp ovaries can provide a clue in preparing maturation diet. Table 2.10 shows the fatty acid profile of some penaeid shrimps compiled from literature. A basic similarity can be observed in the fatty acid composition of different species. Linoleic, eicosapentaenoic and docosahexaenoic fatty acids were found to be present in relatively higher concentrations. Based on the fatty acid profiles of *G. dibranchiata* (Maine blood worm), a successful penaeid maturation dietary supplement in the Western Hemisphere, Lytle *et al.* (1990), suggested a possible balance of n3 and n6 fatty acids in the maturation diets. They proposed a successful maturation dietary supplement should have a similar fatty acid profile and high ratios of n3 to n6 and unsaturates to saturates as that of *G. dibranchiata*. However the present study does not confirm with these

Table 2.10. Selected fatty acid composition (% of total fatty acids) of some penaeids, compiled from literature (wild collected samples).

Fatty acid	*P. japonicus*[1]	*P. indicus*[2]	*P. setiferus*[3]	*P. stylirostris*[4]	*P. vannamei*[4]	*P. kerathurus*[5]
C18: 1 n9	11.9	13.0	15.2	17.5	13.1	12.6
C18: 2 n6	1.5	2.5	NR	3.0	0.9	0.7
C18: 3 n3	0.6	1.1	NR	1.8	0.6	0.3
C20: 4 n6	3.3	6.1	4.1	1.3	4.1	3.2
C20: 5 n3	12.6	9.5	9.9	7.6	5.6	13.5
C22: 6 n3	9.4	11.9	7.0	11.3	3.9	12.4

1-5 References; 1, Guary *et al.* (1974); 2, Read (1977); 3, Middleditch *et al.* (1980a); 4, Araujo (1991); and 5, Luis and Ponte (1993). Values for *P. indicus* and *P. kerathurus* are whole body samples of females, others are late developing ovaries. Great similarity between ovarian fatty acids and whole body fatty acids has been reported by Guary *et al.* (1974).

Table 2.11: Comparison of fatty acid profile of *P. bermudensis* with that of some polychaetes and oligochaetes (compiled from literature).

Fatty acids (% of total)	*P. bermudensis*[1]	*G. dibranchiata*[2]	*A. reesei*[2]	*Neries viridens*[2]	*N. diversicolor*[3]	*Pheretima sp.*[2]	*Lumbricus rubellus*[2]	*Eudrilus euginiae*[2]
C 14: 0	12.93	0.52	6.93	1.12	0.9	2.50	1.62	3.53
C 15: 0	4.22	--	--	--	--	--	--	--
C 16: 0	9.60	8.69	4.13	13.25	17.9	4.98	3.16	4.93
C 17: 0	2.29	--	--	--	--	--	--	--
C 18: 0	6.14	6.45	4.06	5.20	5.6	9.11	8.86	9.88
C 20: 0	--	1.11	0.17	1.31	--	0.56	0.37	0.63
C 22: 0	--	1.97	2.16	0.43	--	1.50	1.02	1.51
C 23: 0	--	--	--	--	--	1.64	1.22	1.47
C 16: 1	4.27	8.61	40.59	8.41	1.0	5.03	3.82	4.86
C 18: 1	11.48	9.96	10.09	12.58	15.8	14.29	14.37	30.94
C 20: 1	6.68	10.69	7.97	9.11	3.8	6.58	9.58	8.13
C 22: 1	--	6.56	4.35	6.09	--	0.05	0.08	0.07

C 24: 1	2.20	--	--	--	--	--	--	--
C16 : 3n3	--	--	--	--	2.8	--	--	--
C 18: 2n6	8.19	0.61	0.48	0.56	13.6	13.47	12.63	15.63
C 18: 3n3	0.62	0.57	0.57	2.10	1.5	0.97	1.77	2.51
C 18: 4n3	1.58	--	--	--	--	--	--	--
C 20: 2n6	--	1.08	1.40	0.66	6.6	4.51	5.60	5.06
C 20: 3n3	--	0.49	0.25	0.11	--	8.64	4.90	4.39
C 20: 4n6	10.01	1.49	1.48	1.34	4.3	12.11	17.31	14.75
C 20: 5n3	2.48	25.44	5.54	25.33	--	10.78	12.51	9.63
C 22: 2n6	--	1.02	0.80	0.56	--	0.32	0.31	0.23
C 22: 3n3	--	2.69	2.76	2.94	--	--	--	--
C 22: 5n3	--	6.91	2.78	5.96	9.0	1.74	0.88	0.71
C 22: 6n3	1.19	5.17	3.47	2.89	1.0	1.19	0.25	0.18
n3/n6	0.32	9.83	3.71	12.60	0.6	0.77	0.57	0.49

1-3 References : 1. Present study ; 2. Lytle et al. (1990) ; 3. Luis and Ponte (1993).

observations. *P. bermudensis* was not rich in n3 PUFA and had a very low ratio of n3 to n6 and unsaturates to saturates, yet when fed as dietary supplement it improved the reproductive performance of *P. semisulcatus* (Table 2.8). Table 2.11 compares the fatty acid profile of *P. bermudensis* with that of some polychaetes and oligochaetes (compiled from literature). The eicosapentaenoic acid and docosahexaenoic acid levels of *P. bermudensis* were found to be very low compared to that of published reports on marine invertebrates like shrimp and polychaetes. The other two n3 fatty acids, C18: 3n3 and C18: 4n3 were present in fairly good concentrations for marine animals. If the statement that EPA and DHA are the active compounds involved in the spawning and egg and larval quality of female shrimp is true, then it is not unreasonable to suggest from the present results that the active compounds involved may be linolenic acid (C18: 3n3). Marine animals are unable to synthesize linoleic acid and linolenic acid, but can convert them from dietary sources into longer PUFA like C20: 4n6, C20: 5n3 and C22: 6n3 (Cowey and Sargent, 1972). Holland (1978) suggested synthesis of longer chain PUFA from C18: 2n6 and C18: 3n3 in marine invertebrates. Kayama *et al.* (1980) demonstrated the ability of *P. japonicus* to convert dietary C18: 3n3 to C20: 5n3 and C22: 6n3 and suggested that crustaceans can metabolize fatty acids through the pathways similar to those of fish, utilizing, depositing and converting the exogenous and endogenous lipids in oceanic food chain. Luis and Ponte (1993) suggested a possible conversion of C18: 2n6 and C18: 3n3 to longer carbon chain unsaturated fatty acids by *P. kerathurus*, when fed a diet of rag worm (*Neries diversicolor*). In *P. vannamei*, adult *Artemia* supplementation was found to reduce the overall n3 PUFA content of the brood stock diet, but at the same time increased maturation performance (Naessens *et al.*, 1997). They concluded that it was not the n3 PUFA content of the diet that enhanced maturation performance.

At the same time *P. bermudensis* was found to be rich in n6 PUFA, especially C20: 4n6 (10%). Tidwell *et al.* (1997) observed a similar fatty acid profile for benthic oligochaetes in shrimp culture ponds, with high concentration of C20: 4n6 and low concentration of C22: 6n3. Dall *et al.* (1991) observed high concentration of C20: 4n6 in the fatty acid composition of natural prey species of *P. esculentus* and suggested that n6 PUFA, particularly C20: 4n6 may be of major importance in shrimp diet. Lilly and Bottino (1981) reported about 9% arachidonic acid in *P. setiferus* lipids.

Arachidonic acid (AA) and other C20 fatty acids are precursors of prostaglandins in many animals (von Euler and Eliason, 1967). In mammals arachidonic acid is the precursor of eicosanoids (prostaglandins and leukotrienes), which are a group of short lived, highly biologically active molecules. The n3 PUFA, principally C20: 5n3 plays a physiological role in modulating the formation of eicosanoids from C20: 4n6 by competing with the enzyme systems converting C20: 4n6 to eicosanoids (Fig. 2.1) (Anon.1992). Sargent *et al.* (1993) established in fishes that biologically active eicosanoids are formed principally from C20: 4n6 and concluded that n6 PUFA are essential nutrients for fish.

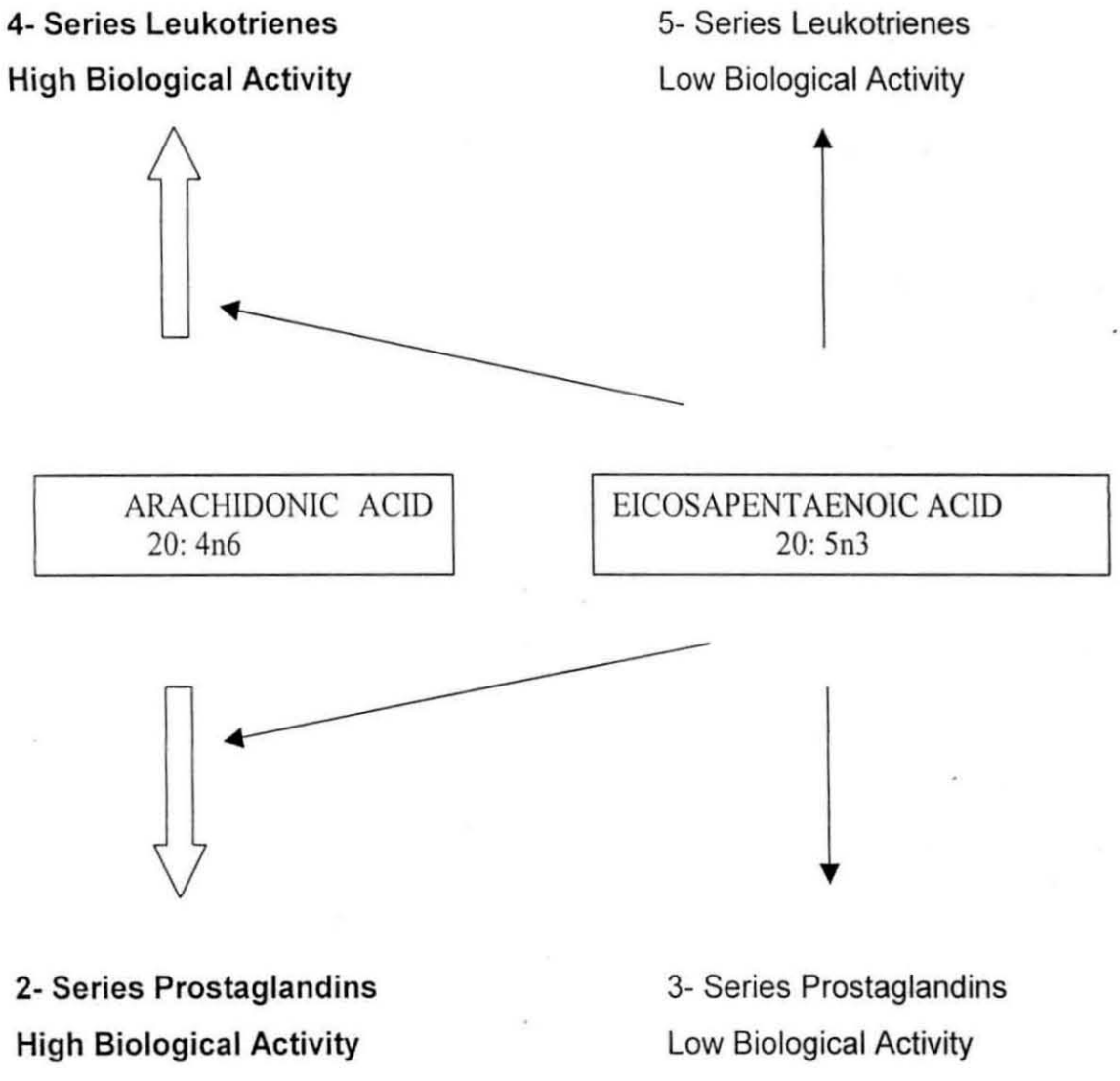


Fig. 2.1: Interaction between AA and EPA in the production of eicosanoids (prostaglandins and leukotrienes). (Source: Anon., 1992).

Endogenous prostaglandins have not been reported in crustaceans (Middleditch *et al.*, 1979 and 1980a). However, in vitro experiments have resulted in the conversion of C20: 3 acid into prostaglandin E_1 in low yield by lobster stomach and gill homogenates (Christ and van Dorp, 1973). Since prostaglandin concentrations are high in human seminal fluid (Bergström and Samuelsson, 1962 and Jonsson *et al.*, 1975 and 1976), these compounds have been implicated in the stimulation of human uterine contractions during labour (von Euler and Eliasson, 1967). Middleditch *et al.*, (1979 and 1980a), suggested that a possible role of C20: 4n6 and C20: 5n3 in the reproduction of shrimp was mediated by prostaglandins. In cod eggs, 18:0/20:4 and 18:1/20:4 were found to be the major molecular species of phophatidylinositol (Bell, 1989 and Tocher and Sargon, 1984). This data strongly points to the essentiality of C20: 4n6 for embryonic and larval development, with a specified role in eicosanoid production. Eicosanoids are important in the control of ovulation (Mustafa and Srivastava, 1989 and Sorbera *et al.*, 1998), embryogenesis, development of immune system, hatching and early larval performance (Bruce *et al.*, 1999). Croz *et al.* (1988) isolated five prostaglandins and three related compounds from the polychaete, *A. reeseii* (Panama blood worm), which is used as a dietary supplement to accelerate ovarian maturation of penaeid shrimps in Central America. The success of *P. bermudensis* as a dietary supplement in inducing maturation and spawning in *P. semisulcatus* and the presence of high concentrations of n6 PUFA, especially C20; 4n6, in the same worm, suggests a possible role of prostaglandins in the reproduction of *P. semisulcatus*. Hamdullah Mustanfi of Qazwin in the *Nuzhat-ul-Qulub*, a scientific encyclopedia written in AD1340, (Stephenson, 1930) reported the property of earthworms to induce immediate delivery in cases of difficult labour. Many others have also reported the property of earthworms in facilitating

delivery (Hussain, 1771; Khan, 1911; Shukla, 1950; Kabiruddin, 1955; Anon., 1959 and Wahid and Siddiqui, 1961). This role of earthworms in facilitating labour, points to the possible presence of prostaglandins or related compounds in them.

The fatty acid analysis of *P. bermudensis* also revealed high concentrations of some saturated and monounsaturated fatty acids (Table 2.8). C14: 0, C16: 0, C18: 0 were the predominant saturated fatty acids, while C16: 1n7, C18: 1n9 and C20: 1n9 were the dominant monounsaturated fatty acids. The neutral lipids (mainly triglycerides) in the ovary of *P. japonicus* were found enriched in 16: 1 and 18: 1 fatty acids during ovarian development. Neutral lipids serve as energy sources for oogenesis and vitellogenesis (Guary *et al.*, 1974 and Teshima *et al.*, 1988). Clarke *et al.* (1990) suggested that saturated and monounsaturated fatty acids may serve as a source of energy during embryogenesis and early larval development in *Macrobrachium rosenbergii*. In fishes 20: 1 and 22: 1 fatty acids are largely catabolised during ovarian maturation to provide the metabolic energy that is necessary for the formation of gonad and eggs (Sargent, 1995). Ward *et al.* (1979) recorded the fatty acid changes during larval development of *P. setiferus*. They observed that the major fatty acids during the egg stage were palmitic (C16: 0), oleic (C18: 0), arachidonic (C20: 4), palmitoleic (C16: 1), stearic (C18: 0) and docosahexaenoic (C22: 6) acids in the order of decreasing concentrations. They observed a general decrease in fatty acid concentration/gm weight (except for DHA) during development from egg to post larvae and suggested that this may be due to the utilization of fatty acid as energy source during egg development and the non-feeding nauplii stages.

Molluscs (squid and clam) were observed to be rich sources of PUFA, especially n3 PUFA (Kanazawa, 1990; Lytle *et al.*, 1990 and Luis and Ponte, 1993). Still the shrimps fed the standard diet that constituted exclusively by molluscs, performed poorly compared to those fed the experimental diet (where molluscs were supplemented by *P. bermudensis*). In this context circumstantial evidence suggest the possible role of arachidonic acid as prostaglandin precursor in the ovarian maturation of *P. semisulcatus*. At the same time a possible role of amino acids of *P. bermudensis* in shrimp maturation cannot be neglected. In the common sole, *Solea solea*, casein was found to act as a nutritive stimulant in inducing maturation and spawning (Flüćhter and Trommsdorf, 1974). Another possible factor in *P. bermudensis* that may trigger reproduction in penaeid shrimp could be of endocrine origin. A number of specific hormones (both inhibitory and stimulatory) were shown to be involved in the reproduction of shrimp (Mzusy and Payen, 1988). Recent researches suggest that oral administration of specific neuropeptides may stimulate maturation in insects and possibly, in crustaceans (Schoofs. Pers. Comm. *vide* Nassens *et al.*, 1997). Yano (1992) detected a vitellogenesis stimulating factor and characterised it as a peptide hormone by fractionating thoracic ganglion extract of vitellogenic females of *P. japonicus*. It is not known whether *P. bermudensis* contain any analogous peptides that could be active in promoting reproduction in *P. semisulcatus*.

Among the three growth stages of *P. bermudensis*, the non-clitellates were found to contain fairly high contents of protein (Table 2.1). The total lipid content of non-clitellates was 11.06%, which was close to the optimum dietary lipid level for shrimp maturation suggested by Bray *et al.*, (1990). During the present experiment non-clitellates constituted about 80-

85% of the total worms fed. Thus it is preferable to selectively harvest the non-clitellates for feeding, while the juveniles and clitellates may be left to build up and maintain their population.

CHAPTER – 3

CULTURE AND BIOLOGY OF THE INTERTIDAL OLIGOCHAETE *PONTODRILUS BERMUDENSIS*

INTRODUCTION

Culture of annelids has been successfully practiced for over many years. Advantageous characters like smaller size, shorter life cycle and considerable reproductive potential made their laboratory culture easy. Annelids were mostly cultivated to serve for assay of pollution. Species like *Capitella capitata*, *Dorvillea articulata, Neanthes arenaceodentata, Neries grubei, Pomatoceros triqueter, Ophryotrocha labronica, O. diaderraa, Dinophilus gyrociliatus* and larvae of *Sabellaria spinulosa* are cultured in laboratories for assessing marine pollution (Reish, 1955, 1957a and b; 1960; 1967; 1970; Reish and Barnard, 1960; Kitamori, 1961; Wilson, 1968a and b; Bellan *et al.*, 1969, 1971, 1972; Åkesson, 1970; Klöckner, 1976 and George, 1975).

Annelids are also famous as food organisms. They are extensively used as fish bait as well as food for cultured carnivorous invertebrates and fishes. They can be mass cultured easily. The lugworms *Arenicola cristata* and *A. marina* are suitable food source for many fish species and crustaceans and are mass cultured as fishing bait and as feed in aquarium and aquaculture farms. Several oligochaetes like the white worm, *Enchytraeus*, the earthworm *Lumbricus* and the small red worm *Tubifex* have been used as food for marine animals. Blount (1937) and Loosanoff (1937) described successful mass culture of white worms. They suggest keeping several small cultures than few larger ones, constant daily care, moist substratum and frequent feeding in small quantity. Swingle (1961)

details the commercial production of red worms as fish bait and food for cultured animals. Several species of Tubificids are used as food for a variety of invertebrates, fishes and frogs. Culture of *Tubifex hattai* in an artificial medium has been described by Inase (1960 a and b). Production of *T. tubifex* in different culture media have been discussed by Kosiorek (1974); Marian and Pandian (1984 and 1985); Marian *et al.* (1989); Mollah and Ahamed (1989); and Ahamed and Mollah (1992). Commercial production of the polychaetes, *Glycera dibranchiata* (Maine blood worm) and *Americonophus reeseii* (Panama blood worm) is practiced in U.S.A. The common earthworm *Lumbricus terrestris* is a preferred food for cultured marine invertebrates. It can be cultured with soil or without it (Hess, 1937).

Earthworms belong to five families - Moniligastridae, Megascolicidae, Eudrilidae, Glossoscolecidae and Lumbricidae – of class Oligochaeta, and have a worldwide distribution (Jameison, 1971). Most of the earthworms are terrestrial organisms, which inhabit the soil. Earthworm helps in composting organic matter and maintains soil fertility. Several species of earthworms are cultured for organic waste recycling and for improving soil fertility. *Eisenia foetida* (Hartenstein *et al.*, 1979 a and b). *Eudrilus euginae* (Kale and Bano, 1988); *Lampito mauritii* (Ismail, 1993; 1997; Anne Grace and Ismail, 1995; and Anne Grace, 1996); and *Perionyx excavatus* (Ismail, 1997) are the main species of terrestrial earthworms cultured for composting. Culture of earthworms is known as vermiculture and the technology used for this as vermitech. Many authors have discussed the culture of earthworms and its usage and importance in agriculture (Bhawalkar, 1992; Bhiday, 1994; Purakayastha and Bhatnagar, 1997; Ismail, 1997; and Rao, 1998).

Though most of earthworms are terrestrial and soil dwelling organisms, five species of the genus *Pontodrilus- P. bermudensis, P. litoralis, P. gracilis, P. matsushimensis* and *P. phosphorus* – have been reported from the littoral regions of different parts of the world (Michaelson, 1900, 1910; Beddarad, 1895, Stephenson, 1915b and Gates 1943). Among these *P. bermudensis* Beddard has a worldwide distribution in the tropical, sub tropical and warm tropical regions of the Atlantic, Pacific and Indian Ocean. Its distribution extends to 45° N and 45° S of the equator with particular abundance in the tropical and subtropical belt (Subba Rao and Ganapati, 1975). In India, Stephenson (1914) reported the worm from Chilka Lake, where almost fresh water conditions prevail from July to September and varying ranges of salinity from 10‰ to 32‰ during the rest of the year. The worm has also been reported from intertidal regions in Pamban, Port Blair (Andamans), Laccadives, Maldives, Kovalam, Port Okha (Gulf of Kutch) and Elephanta (Beddard, 1903; Stephenson, 1914; 1915a; 1916,1930, Aiyar, 1929; Gates, 1936; and Menon and Sareen, 1967).

P. bermudensis exhibits a wide range of salinity tolerance from 5‰ to 33‰, with optimum at 25‰ (Ganapati and Subba Rao,1972). The worms generally prefer decaying and half decaying seaweeds, under stones, rotten logs etc., where relatively high carbon content is available. Subba Rao and Ganapati (1974 and 1975) studied the bionomics of *P. bermudensis* from the brackish water areas of the Visakhapatnam Harbour. They observed that the habitat of the animals was subjected to wide salinity fluctuations and heavy domestic and industrial pollution. Sexually mature worms with well-developed clitellum were observed by October –November. Cocoon shedding was from the end of November till late May. The cocoons were found to lay free in the sediment, under surfaces of the pebbles and detritus matter and sides of the rocks.

Stephenson (1915a) observed that in Chilka Lake, mature worms were present only in late winter and early spring when the salinity ranged from 10‰ to 32‰. During the fresh water season from July to September, mature forms were absent. Panikkar and Aiyar (1939), collected the mature forms during the months of December, January and February from the brackish waters of Adyar where salinity ranged from 16.9‰ to 30.44‰.

Cocoons of *P. bermudensis* were observed to measure 3-7 mm in length and were milky gray in colour first, which soon changed to green and later to deep pink. Cocoons were spindle shaped and the number of eggs varied from 1-6. A thick viscous albuminous fluid inside the cocoons provides nourishment for the developing worms. (Subba Rao and Ganapati, 1975).

As a dietary supplement, *P. bermudensis* was found to induce ovarian maturation and spawning in *P. semisulcatus* in the present study. Biochemical analysis revealed the worm to be rich in protein and lipids. In the light of this, attempts were made during the present study to culture *P. bermudensis* in laboratory. Ganapati and Subba Rao (1972) in their salinity tolerance studies were able to maintain *P. bermudensis* in large glass troughs filled with filtered brackish water for 96 hours. Prior attempts to culture *P. bermudensis* in land based culture systems for a prolonged period has not been reported. Present chapter describes the attempts to culture these worms in petridishes with agar media, in trays and in wooden boxes with sand substratum and different organic amendments. Long term culture of the worms with periodic harvesting, to ascertain the harvestable biomass and sustainability of the population was also tried.

MATERIALS AND METHODS

Collection of worms:

Pontodrilus bermudensis were collected from the intertidal area of Gulf of Mannar behind the shrimp hatchery of CMFRI, Mandapam Camp. The worms were cleaned, counted and weighed before transferring to the culture systems. As the worms were very delicate they were handled carefully.

Experiment 1. Culture in Petridish

Petridishes of 10 cm diameter and 1.5 cm height were used. Culture media was agar. The experiment was done in triplicate. About 500 mg of agar (obtained from the Algology Department of MRC of CMFRI) was weighed and dissolved in 100 ml seawater and poured into the petridishes. It was allowed to cool for one day. Ten juveniles of *P. bermudensis* were introduced into each petridish and closed with lid. Petridishes were kept in dark. Observations were made once in every five days.

Experiment 2. Culture in Trays

Plastic trays of 40 x 30 x 5 cm size were used for the culture. Twelve trays (four experimental setups – each conducted in triplicate) were perforated at the base to facilitate drainage of water. A fine mesh cloth lining on the bottom of the tray prevented escape of worms through the holes. In the trays a sand layer (seashore sand of 2cm thickness) was spread above a ½ cm thick coarse sand layer. Twenty non-clitellate

(immature) worms, whose biomass was recorded, were introduced into each of the trays.

Approximately 150 g fresh cow dung was added to each of the trays, followed by seaweeds (500 g) in tray 1, leaf liter (500 g) in tray 2 and hay (500 g) in tray 3. Tray 4 was kept as control with only 150g of cow dung. Each of the treatments had three replicates and data for worm biomass was recorded on day 30 and 60 from inoculation. The trays were regularly sprinkled with seawater during the period of observation and were covered with moist gunny cloth to retain moisture. The trays were kept in a dark and damp room.

Experiment 3. Culture in wooden crates

Rectangular wooden crates (45cm x 36cm x 30cm) made by wooden flanks were used for culture. Inner sides of the boxes were lined with fine mesh cloth. A vermibed was prepared in each of the boxes following the method of Ismail (1993 and 1997). A layer of coarse sand of thickness 5-6 cm followed a basal layer of broken bricks and pebbles to ensure proper drainage. A layer of intertidal sand upto a height of not less than 12cm after moistening topped this. Hundred non-clitellate worms, whose biomass was recorded, were inoculated into the top sand layer.

About 500gms of fresh cow dung was mixed with 100gms of granite soil (to control infestation of fly larvae, Dr. Uday Bhawalker personal communication) and scattered over the soil layer, followed by seaweeds (1 Kg) in crate 1 and leaf litter (1 Kg) in crate 2. Crate 3 was kept as control with only 500g of cow dung and without any organic amendment. Each treatment was done in triplicate and worm biomass was recorded on day 30, 80 and 200 from inoculation. Seawater was sprinkled regularly

during the period of experiment and the crates were covered with moist gunny cloth to retain moisture. Crates were kept in shade. The diagrammatic representation of a Vermibed or worm bed followed in the present study is given in Fig. 3.1. Plate 3.1 shows the culture units of *P. bermudensis*.

Experiment 4. Culture in wooden crates with periodical harvesting

This experiment ascertained the harvestable biomass and the sustainable capacity of the remaining population of *P. bermudensis* in culture system. The experiment was conducted in duplicate. Vermibed was prepared in a similar manner as described for experiment 3. Hundred non-clitellate worms were introduced into the sand layer after recording biomass. About 500g of fresh cow dung was mixed with 100g of granite soil and spread over the sand layer. 500gms each of seaweeds and leaf litter were added to both the units. The units were watered regularly during the experimental period. The units were covered with moist gunny cloth. From the day thirty-first onwards 25g each of fresh cow dung, seaweeds and leaf litter were spread as a thin layer on top once in every 10 days up to the day sixty. Thereafter only watering was continued. The top layer of decaying organic matter (mulch) was turned gently using a fork periodically.

By the day ninety the population was randomly sampled to estimate the harvestable biomass. On the day of harvesting the whole unit was watered profusely, so that the worms moved to the upper layer. The top layer of mulch and sand was collected separately and worms were hand picked. The total biomass or the standing biomass of the unit on the day of harvest was recorded. Only the non-clitellate worms were harvested and

Fig. 3.1: Diagrammatic representation of the Vermibed prepared in the present study.

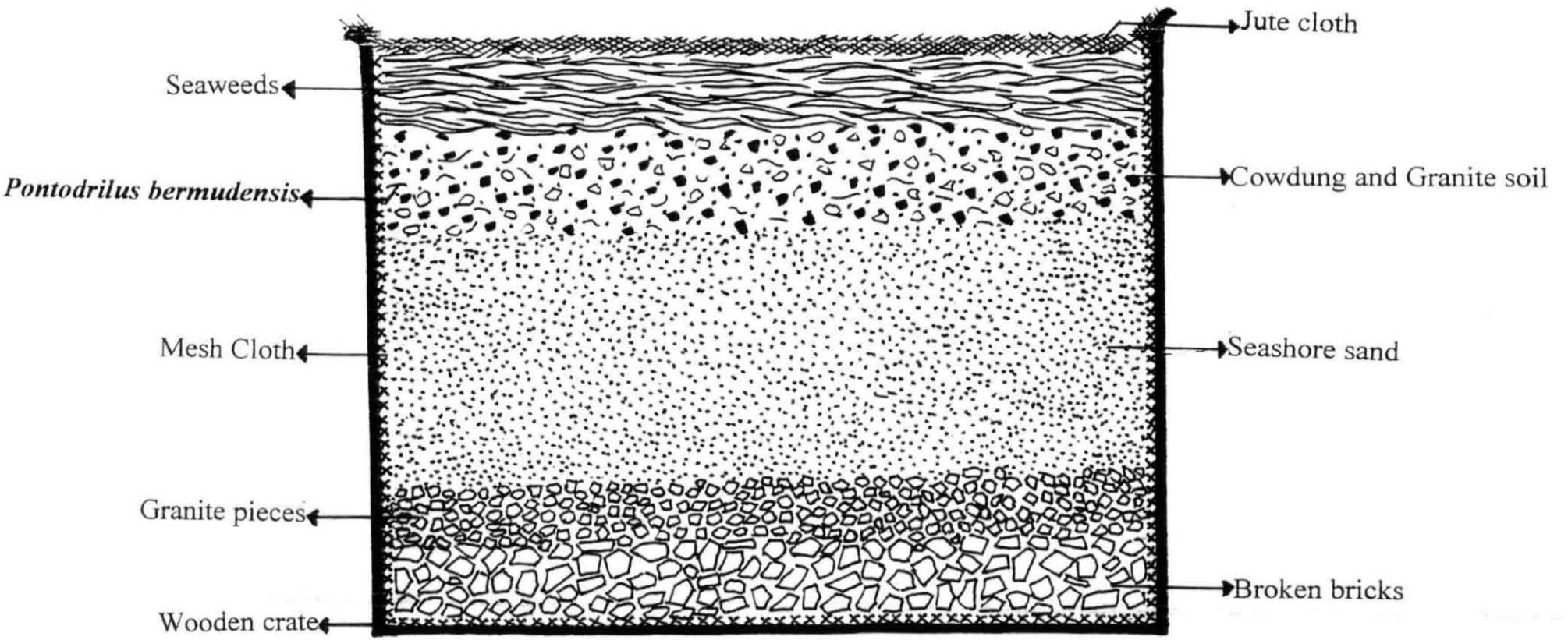


Plate 3.1. Culture of *P. bermudensis* in wooden crates

the juveniles and clitellates were continued in into the unit to build up the population. Harvested worms were weighed and biomass was recorded. Crates were reloaded and the process repeated.

Inoculation of worms to unit 2 was done after 30 days of inoculation to unit 1 to facilitate harvesting of worms from the two units alternately.

Studies on growth stages.

In experiment 4 at the time of harvest the length, weight and appearance of the different growth stages of *P. bermudensis* were recorded.

RESULTS

Experiment. 1. Culture in Petridishes

Juvenile worms inoculated to the agar medium were healthy and were observed to crawl along the sides and into the media. But within 15-20 days all the worms in petridishes were found dead. No microbial growth was observed on the plates and the agar media was almost intact. Further attempts to culture the worms in agar media was not undertaken.

Experiment 2. Culture in trays

The results of the experiment are given in Table 3.1 and 3.2. In tray 1, 2 and 4, juvenile worms appeared by the day 30. Most of the inoculated non-clitellates were dead. The few surviving ones were in the mature, clitellate stage. In tray 3 with hay amendment, all the worms were found

Table 3.1: Tray culture of *P. bermudensis*: Population (No.s Mean + SE) in sand with different amendments.

Sl. No.	Amendment	Population in Number		
		Day 0	Day 30	Day 60
1.	Sand + cow dung + seaweeds	20 ± 0	76.0 ± 6.24	0
2.	Sand + cow dung + leaf litter	20 ± 0	66.0 ± 6.98	0
3.	Sand + cow dung + hay	20 ± 0	0	0
4.	Sand + cow dung	20 ± 0	41.7 ± 4.06	0

Table. 3.2: Tray culture of *P. bermudensis*: Biomass (g Mean + SE) in sand with different amendments.

Sl. No.	Amendment	Biomass in (g)		
		Day 0	Day 30	Day 60
1.	Sand + cow dung + seaweeds	20.31 ± 0.84	21.62 ± 1.24	0
2.	Sand + cow dung + leaf litter	19.35 ± 0.08	20.30 ± 1.62	0
3.	Sand + cow dung + hay	19.78 ± 0.70	0	0
4.	Sand + cow dung	19.65 ± 0.53	15.15 ± 2.45	0

dead by the day 30. By the day 50 the culture medium in all the trays was found infested with the larvae of some insects in spite of treatment with neem oil. All the worms were found dead by day 60 and the culture was terminated.

Experiment 3. Culture in wooden crates

Table 3.3 and 3.4 summarize the results of the experiment. When compared the growth (live weight per worm) recorded on the day 80, both seaweed and leaf litter amendments significantly promoted than the control of cow dung only. But no significant difference in growth was observed between the seaweed and leaf litter amendments (Table 3.5). Maximum values of biomass and population numbers were recorded 80 days after the inoculation of worms. There was about 6.14 times increase in biomass in seaweed amendment and 5.77 times in leaf litter amendment by the 80^{th} day. Population numbers recorded an increase of 40.66 times and 39.88 times for seaweed and leaf litter amendments respectively by the 80^{th} day. A decline in biomass and population numbers was evident after 80 days (Table 3.3 and 3.4 and Fig. 3.2 and 3.3).

Experiment 4. Standing biomass and harvest

Based on the observations on the culture in wooden crates it was proposed to harvest the unit first time after 90 days of inoculation, and thereafter once in every 30 days (Fig 3.4). An average of 452.8gm of worms were harvested. Different stages of worms were sorted out and only non-clitellate worms were harvested whereas, juveniles and clitellates

Table. 3.3: Mass culture of *P. bermudensis* in wooden crates: Population (No.s Mean + SE) in sand with different amendments

Sl. No.	Amendment	Population in Number			
		Day 0	Day 30	Day 80	Day 200
1.	Sand + cow dung + seaweeds	100 ± 0	3122.00 ± 34.17	4021.33 ± 186.57	594.67 ± 10.93
2.	Sand + cow dung + leaf litter	100 ± 0	3073.67 ± 37.15	3947.00 ± 116.84	548.33 ± 22.58
3.	Sand + cow dung (control)	100 ± 0	2275.67 ± 45.19	2697.33 ± 16.79	321.67 ± 12.86

Table. 3.4: Mass culture of *P. bermudensis* in wooden crates: Biomass (No.s Mean + SE) in sand with different amendments

Sl. No.	Amendment	Biomass in g.			
		Day 0	Day 30	Day 80	Day 200
1.	Sand + cow dung + seaweeds	98.62 ± 0.26	152.29 ± 7.76	577.20 ± 21.37	123.20 ± 2.25
2.	Sand + cow dung + leaf litter	98.85 ± 0.55	144.39 ± 2.48	539.37 ± 14.03	127.46 ± 10.23
3.	Sand + cow dung (control)	98.52 ± 0.25	122.64 ± 9.26	241.99 ± 2.25	90.58 ± 9.21

Table 3.5: Analysis of Variance of growth (live weight per worm) of *P. bermudensis* in the three amendments on day 80.

Summary

Group	*Count*	*Sum*	*Average*	*Variance*
Amendment 1 (sand + Cow dung + seaweeds)	3	0.433717	0.144572	0.000374
Amendment 2 (sand + Cow dung + leaf litter)	3	0.410590	0.136863	6.86E-05
Amendment 3 (cow dung)	3	0.269204	0.089735	7.49E-06

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*Fcrit*
Between Groups	0.005287735	2	0.002644	17.60313	0.003087	5.143249
Within Groups	0.000901158	6	0.00015			
Total	0.006188893	8				

Amendments 1 and 2 are significantly different from amendment 3 (control).

Fig. 3.2: Population density of *P. bermudensis* cultured in wooden crates

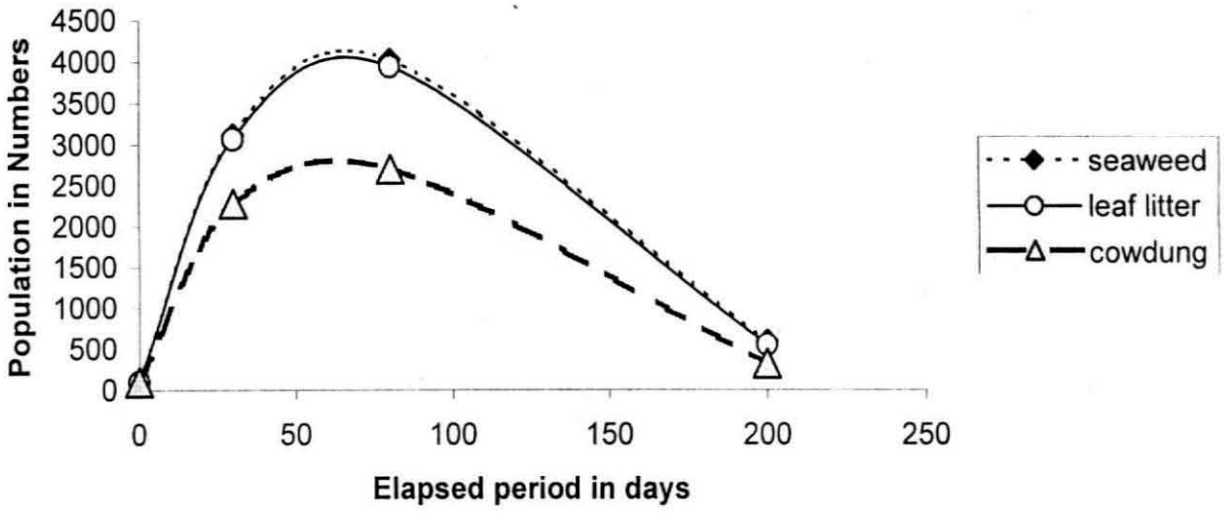


Fig. 3.3: Biomass of *P. bermudensis* cultured in wooden crates

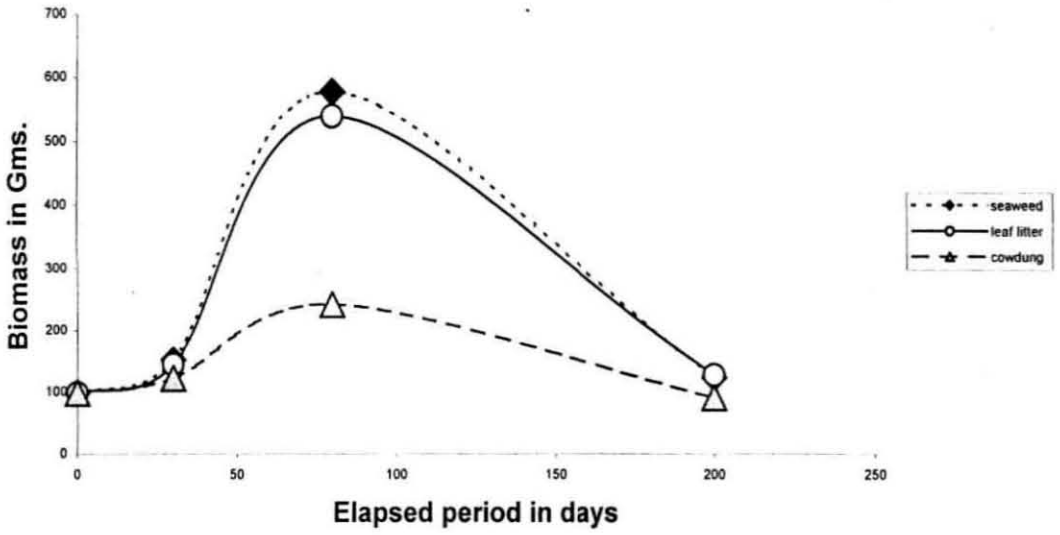
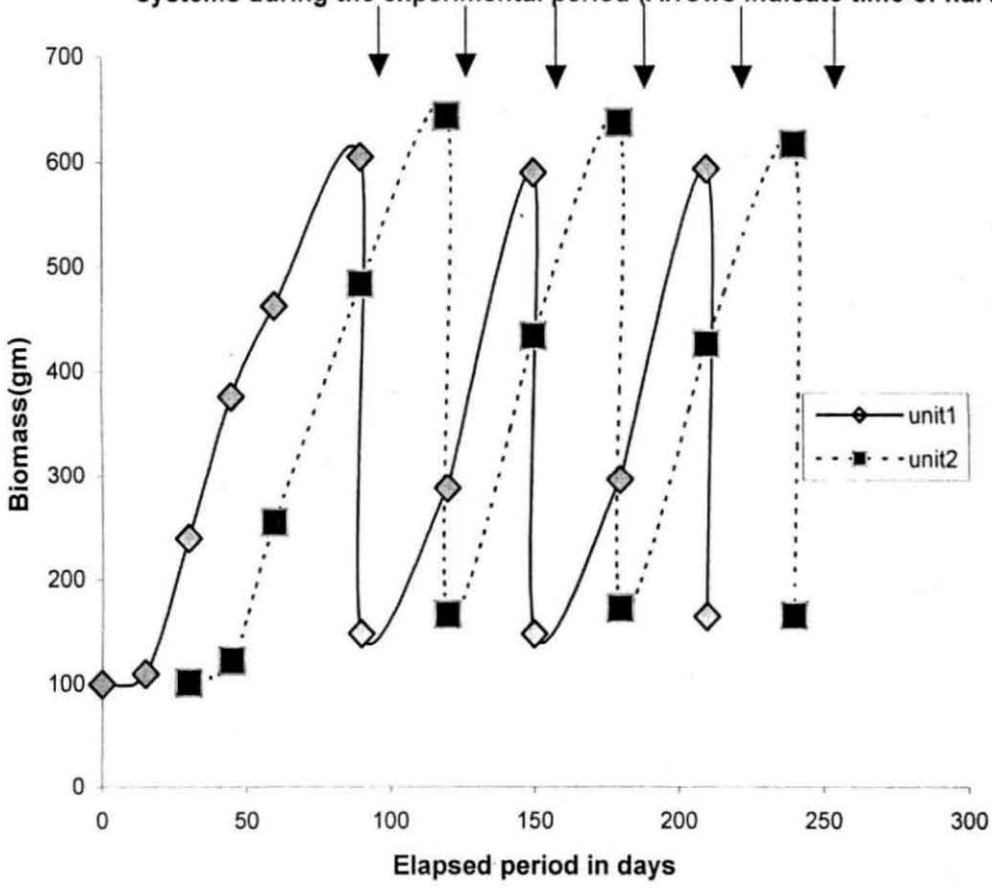


Fig. 3.4: Standing biomass and yield of *P. bermudensis* in twin culture systems during the experimental period (Arrows indicate time of harvest)



were left to build up the population. On the day 90 standing biomass in the unit 1 was 604.77gm of which 456.41gm was harvested. On day 120, which is after 90 days of inoculation of unit 2, the standing biomass of this unit was 643gm, and 477.09gm was harvested from it. The standing biomass of unit 1 increased to 589.83gm on the day 150, of which 441.76gm was harvested. On the day 180, the standing biomass of unit 2 recovered to 636.41gm, from which 464.17gm was harvested. By day 210 the biomass of unit 1 was 592.83gm and 427.18gm was harvested. When the standing biomass of unit 2 recovered to 615.86gm on day 240, 450.72gm was harvested.

Studies on growth stages:

Plate 3.2 shows the three stages in the life cycle of *P. bermudensis* from the culture units.

The juveniles or the young ones hatching from the cocoon ranged from 0.9cm to 1.1 cm in length and weigh around 6 mg on average (Plate 3.3). They were white in colour and were usually found either entwined to the body of clitellates or in groups of 10-15 worms attached to decaying twigs or leaves. The non-clitellates are the transition forms from juveniles to mature worms. They are yet to develop a clitellum and their colour ranges from light pink to dark pink. Juveniles when reach about 3.7cm changes to non-clitellates with light pink colour. The non-clitellates ranged between 3.7 and 7.5cm in length and 150-850mg in weight. The mature or clitellate worms were longer than 7.5 cm (Plate 3.4). The maximum length observed was 12cm. They were dark pink in colour with a brownish tinge. They have a characteristic clitellum between 8^{th} and 19^{th} segment. The

Plate 3.2. Cultured *P. bermudensis* – 3 stages

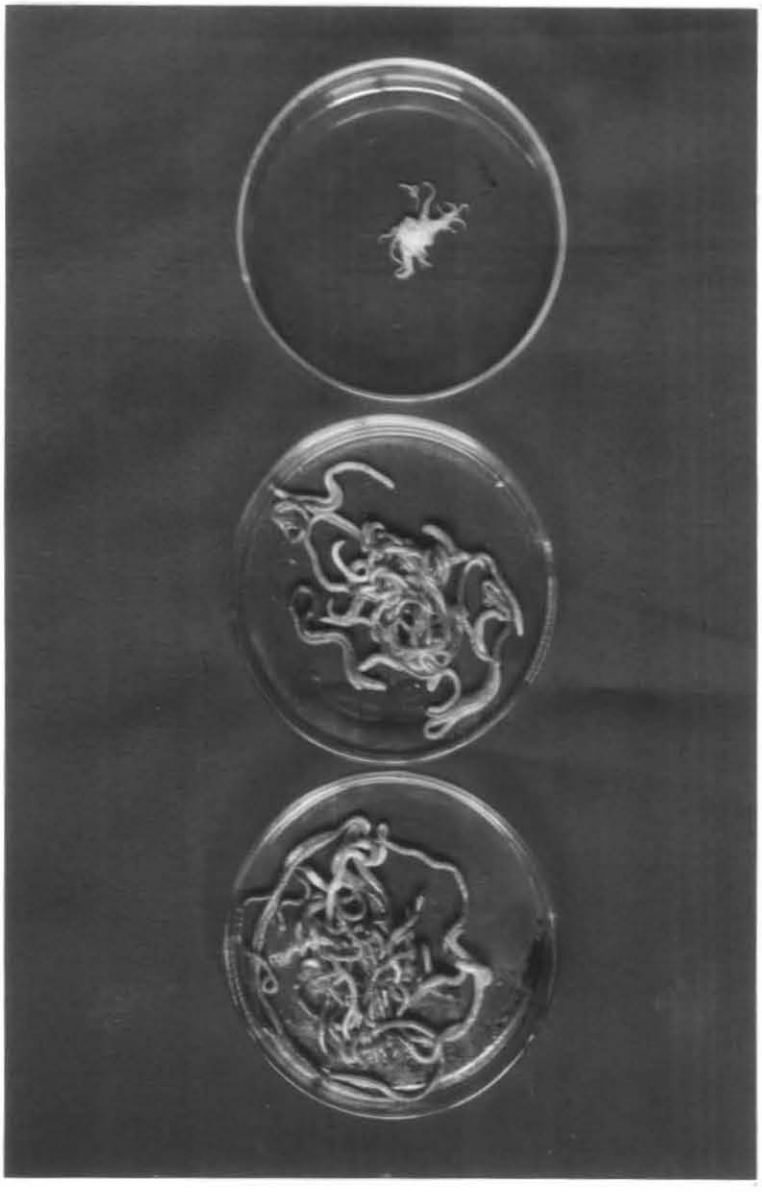


Plate 3.3. Cultured juvenile of *P. bermudensis*

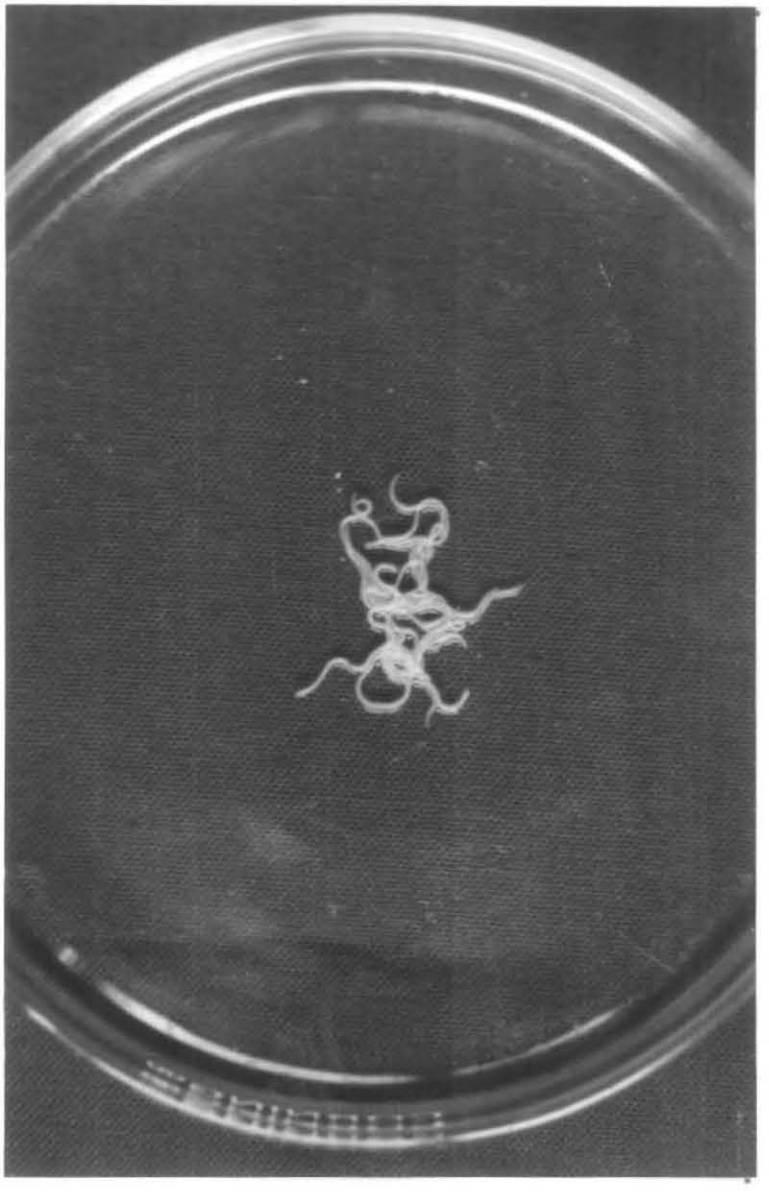


Plate 3.4. Cultured clitellates of *P. bermudensis*

clitellar segments were observed to be very pale in colour and thickened. By the time of shedding, the clitellum was found to attain a yellowish tinge.

DISCUSSION

The present chapter details the attempts on culture of the intertidal oligochaete *P. bermudensis.* Successful long-term culture was achieved when the worms were reared in wooden crates. George, (1975) described the culture of benthic polychaetes in petridishes with agar media. He found that the agar media, apart from giving good production of worms, offers a transparency through which the growth of worms can be assessed clearly without disturbing them. In the present study, *P. bermudensis* juveniles inoculated to the agar media survived only for 15-20 days. Successful cultures of *L. mauritii* (Anne Grace and Ismail, 1995) and *E. albidus* (Blount, 1937; Loosanoff, 1937) in small containers have been reported. In the present study *P. bermudensis* cultured in plastic trays were found to produce first generation of juveniles in the seaweed and leaf litter amendments and also in the control. In the hay amendment the worms were found dead within one week of inoculation. All the cultures were discarded after 60 days due to infestation of larvae of some insect. But the study proved that seaweed and leaf litter amendments were favourable media for culture. Cow dung alone as organic media also seemed to suitable to produce juveniles but was comparatively less favourable than the other two amendments (Table 3.1 and 3.2).

Mass culture of tubificids in outdoor cement culverts have been described by Marian and Pandian (1984) and Ahamed and Mollah (1992). Hess (1937) reported the culture of the common earthworm, *L. terrestris*, a preferred food item for cultured marine invertebrates, in large boxes with

sand substratum. The worms were observed to grow well in organic amendments like leaf loam or pre-cooked cereal, bread crumbs and corn meal. Organic matter was spread on top every two to three weeks. Hess (1937) also described a similar culture of the faecal earthworm, *Eisenia foetida* with rotten cow and horse manure as organic matter. Swingle (1961) detailed commercial fish worm production in out door wooden or concrete culture beds without soil substratum. Decaying feed provided a peat-like substratum on which the worms thrived. According to Swingle, this was superior to soil as worms were easier to harvest and came out clean. The optimal protein content of the feed was suggested to be between 10-12%.

In the present observation *P. bermudensis* when cultured in wooden crates were found to survive and reproduce for a long period, even after 200 days of inoculation (Table 3.3 and 3.4). Though the worms were found to grow in all the three experimental set-ups, significantly better growth was observed in the two organic amendments (seaweed and leaf litter) than the cow dung alone treatment (control). *P. bermudensis* was observed to prefer seaweed amendment, as evident from the highest values of biomass as well as population number from this amendment. In its natural habitat also *P. bermudensis* was found to aggregate in large numbers under decaying seaweed beds along the Gulf of Mannar and Palk Bay coast. At the same time leaf litter amendment also promoted growth and was not significantly different from seaweed amendment. From the day of inoculation to day 80 a gradual increase in population number and biomass was observed. A decline in biomass and number was observed after day 80, probably due to the utilization of organic matter (Fig. 3.2 and 3.3). Anne Grace and Ismail (1995) and Anne Grace (1996) observed a similar trend in laboratory cultured *L. mauritii.*

Experiment 4 determined harvestable biomass of *P. bermudensis* at a fixed interval from the continuous culture units (Fig. 3.4). The twin unit system used in the experiment permitted periodical harvesting of adult non-clitellates, without adversely affecting the total population. The standing biomass was found to recover within 60 days in a culture system. As the two units were harvested alternately, required time was provided to replenish the standing biomass of the culture units. Similarly a Multi-unit system can be successfully employed for the commercial culture of *P. bermudensis* to supply for daily feeding of broodstock of penaeid prawns in the hatchery. The number of vermiculture units can be increased depending on the requirement of worms. An average of 452.8gm worms were harvested from the two culture units(45x36x30 cm) every 30 days. Marian and Pandian (1984) studied the standing biomass and harvestable yield of *T. tubifex*. The standing biomass of *T. tubifex* was found to recover within 20 days. Ismail (1997) suggested a twin unit system for the proper harvesting of vermicompost through vermitech.

Observations on the breeding period and structure of cocoons of *P. bermudensis* from the brackish water areas of Visakhapatnam Harbour was reported by Subba Rao and Ganapati (1974 and 1975). Information on the three growth stages in the life cycle of *P. bermudensis* was not available. Ismail (1997) described the life cycle pattern of *L. mauritii* and *P. excavatus*. The juveniles of *L. mauritii* was found to measure 0.8-1.5mm in length and weigh around 7mg while that of *P. excavatus* was 3-4mm and 5mg respectively. Juveniles of *L. mauritii* were light brownish – red in colour and those of *P. excavatus* were dark pink in colour. Non-clitellates of both the species were longer than 4cm. Clitellates of *L. mauritii* were 20-25cm in length and 3.5gm in weight and were dark brown coloured. Clitellates of *P. excavatus* were found to grow up to15cm and were dark bluish-purple in colour. In the present observation juveniles of *P.*

bermudensis were white in colour, which gradually changed to pink at non-clitellate stage and to dark pinkish brown at clitellate stage (Plate 3.2). Length of juveniles ranged from 0.9-3.7cm and that of non-clitellates from 3.7-7.5cm. The clitellates were longer than 7.5cm and were found to grow up to 12cm.

Based on the results of the present culture experiments a timely plan for the commercial culture of *P. bermudensis* is presented in Table 3.6. A multi-unit vermiculture system can be established as part of shrimp hatchery with minimal expenditure.

Though the worm was found to grow well in wooden crates with different organic matter, the effectiveness of cultured worms in inducing maturation in penaeids as dietary supplement was not studied in the present study. Whether any difference exists between the composition of cultured worms and that of wild worms has to be studied. Since diet influences the biochemical composition of the cultured organism, studies to assess the possible difference in the composition of *P. bermudensis* grown in seaweed plus cow dung media and leaf litter plus cow dung media; and to evaluate the most suitable culture media that gives similar biochemical composition of wild worm have to be conducted. The worms are found abundantly under decaying seaweed beds in the intertidal region. Probably seaweed amendment media may give suitable biochemical composition similar to that of wild worm.

Another aspect that has to be considered before introducing these worms, as commercial maturation feed is the possible bioaccumulation of toxic materials in their body. Earthworms were reported to be capable of accumulating toxic residues particularly of metals and agrochemicals. Bioaccumulation of lead, cadmium, chromium, copper, nickel, mercury and zinc in earthworm tissues have been reported (Gish and Christensen,

Table 3.6: Timetable for proper harvesting of *P. bermudensis* by setting up a multi- unit system

Day	Unit I	Unit II	Unit III	Unit IV
0	Vermibed	____	____	____
15	____	Vermibed	____	____
30	Start loading	____	Vermibed	____
45	_____	Start loading	_____	Vermibed
60	Stop loading (only watering)	____	Start loading	____
75	_____	Stop loading	_____	Start loading
90	Harvest/Reload	____	Stop loading	_____
105	_____	Harvest/Reload	_____	Stop loading
120	Stop loading	_____	Harvest/Reload	____
135	_____	Stop loading	_____	Harvest/Reload
150	Harvest/Reload	____	Stop loading	____
165		Harvest/Reload	_____	Stop loading
180			Harvest/Reload	____
195				Harvest/Reload

1973; Hartenstein *et al.*, 1980; Beyer, 1981; Ireland, 1983 and Joseph *et al.*, 1992). Subba Rao and Ganapati (1975) observed *P. bermudensis* to flourish in the heavily polluted areas of Visakhapatnam Harbour. The worms were found to survive even in industrially polluted areas. The intertidal areas of Gulf of Mannar at Mandapam from where the worms were collected for the present study were affected with domestic pollution. So it is essential to monitor any bioaccumulation of metals and agrochemicals in *P. bermudensis* before introducing the worms in culture system as well as using as diet in commercial shrimp maturation systems.

The salient results of the present research work can be summarized as follows:

- The intertidal oligochaete, *P. bermudensis* was observed to significantly improve the reproductive performance of *P. semisulcatus*, when given as dietary supplement. The control group of *P. semisulcatus* fed the standard diet (squid and clam) performed significantly poorer to the test group, for which the standard diet was supplemented with *P. bermudensis*. Significant increase in the average number of spawns, eggs and nauplii per female and average number of eggs and nauplii per spawn were recorded for the test females. The average rate of spawning and egg production was also higher for the test group than that of the control group.
- Maximum maturation response was observed when the worm component formed about 9% of the total feed. At this stage all the females in the tested group were found to respond for induced maturation.
- Among the test group females, individual differences in the response to induced maturation were observed. On the basis of the present study it is suggested that the genetic make up of the female shrimp may be the deciding factor for influencing reproductive

performance than the female size. More studies in this respect are necessary to clarify this point.

- Biochemical analysis of the worm, *P. bermudensis* revealed significant differences in the composition of various metabolic components among the three growth stages. Of the three stages, the non-clitellates were found to be suitable for feeding, as their protein content was comparatively high (51%) and lipid level was at optimum (11.6%). Selective harvesting of worms at this stage will help to sustain the population in culture as well as in natural habitat.
- The fatty acid profile of *P. bermudensis* was observed to differ from that of marine invertebrates, which are generally used as successful shrimp maturation dietary supplement. A preponderance of n6 fatty acids, especially C20: 4n6 (Arachidonic acid) and low values of eicosapentaenoic and docosahexaenoic acids were recorded. Among the n3 fatty acids, C18: 3n3 and C18: 4n3 were found to be present in fairly good quantities. This points to the possibility of bioconversion of linolenic acid to eicosapentaenoic and docosahexaenoic acids in the shrimp ovary. Presence of large amount of arachidonic acid (a precursor of prostaglandins) in the worm, suggests a possible role of prostaglandins in shrimp maturation and spawning. Saturated and monounsaturated fatty

acids which may serve as a source of energy during embryogenesis and early larval development were also present in good amounts.

- *P. bermudensis* was found to grow well in wooden boxes with seashore sand as media with cow dung and seaweed or cow dung and leaf litter as organic amendments. Addition of granite soil prevented infestation of fly larvae and improved growth of the worms. Seaweed amendment was found to give maximum increase in number and biomass.
- The standing biomass of the worms was observed to recover within 60 days after partial harvesting of the total biomass (selective harvesting of non-clitellates) from the culture units. Based on these experiments multi-unit vermiculture system with periodical harvesting and loading of organic matter is proposed as part of shrimp maturation systems with minimal investment to facilitate continuous supply of worms.

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